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Nucleotide metabolism and its control in lactic acid bacteria

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Abstract

Most metabolic reactions are connected through either their utilization of nucleotides or their utilization of nucleotides or their regulation by these metabolites. In this review the biosynthetic pathways for pyrimidine and purine metabolism in lactic acid bacteria are described including the interconversion pathways, the formation of deoxyribonucleotides and the salvage pathways for use of exogenous precursors. The data for the enzymatic and the genetic regulation of these pathways are reviewed, as well as the gene organizations in different lactic acid bacteria. Mutant phenotypes and methods for manipulation of nucleotide pools are also discussed. Our aim is to provide an overview of the physiology and genetics of nucleotide metabolism and its regulation that will facilitate the interpretation of data arising from genetics, metabolomics, proteomics, and transcriptomics in lactic acid bacteria.

Keywords: Nucleotides; Deoxynucleotides; Nucleosides; Nucleobases; Purines; Pyrimidines; Nucleotide salvage; Nucleotide biosynthesis; Control analysis; PurR; PyrR; Gene expression; Enzymes; Allosteric control

Contents

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1. Introduction

Nucleotides are obligatory metabolites in all organisms. They are substrates for RNA and DNA synthesis, and serve as the main energy donors for cellular processes. Some nucleotides are constituents of coenzymes, while others are used for activation of precursors in polysaccharide and lipid synthesis. In addition, nucleotides serve an important role in the regulation of numerous cellular processes from the metabolic level to the level of gene expression. In full-genome microarray experiments and in proteome analysis, the major task after coping with the technical hurdles, is an evaluation of the importance of unexpected regulation patterns, e.g., a three to 10-fold reduction in the amount of the pyrimidine biosynthetic enzymes upon growth of a *Lactococcus lactis* strain on lactose compared to glucose grown cells [1]. The scope of the present review on the network of nucleotide metabolism is therefore 2-fold. Primarily we want to extract as much knowledge as possible about the nucleotide metabolism in lactic acid bacteria. This overview includes the phenotypes of various mutants as an aid in the identification and analysis of mutants, e.g., obtained by pGhost::IS*S1* mutagenesis in stress research [2,3]. Second, by including important auxiliary reactions, such as the dependence of purine biosynthesis on the folate C1 interconversion [4] and formate availability, the review is aimed at building physiologically relevant bridges between separate areas.

Most bacteria are able to produce nucleotides de novo, while others, including some lactic acid bacteria, require addition of either purines or pyrimidines to the growth medium. These auxotrophic bacteria utilize salvage pathways for conversion of the required nucleobases or nucleosides to nucleotides. Many bacteria can utilize nucleotides as sources of purines or pyrimidines,

but these have to be dephosphorylated by extracellular nucleotidases before entering the cell. Prototrophic bacteria also contain elaborate salvage systems for the uptake and utilization of nucleobases and nucleosides. In these bacteria the de novo synthesis pathway is usually silenced under conditions of excess purine or pyrimidine sources, by a combination of protein inhibition and genetic regulation.

Many lactobacilli are auxotrophic for both purines and pyrimidines, and some have an additional requirement for deoxynucleoside because they are unable to reduce the ribonucleotide to the corresponding deoxyribonucleotide for DNA synthesis. They possess a special salvage system based on a *trans-N*-deoxyribosylase and require a deoxynucleoside in combination with purine and pyrimidine bases for growth [5].

The requirement of purine auxotrophic lactic acid bacteria cannot be satisfied by growth in milk. This conclusion was reached from the inability of purine requiring mutants of *L. lactis* and *Streptococcus thermophilus* to grow in milk [6,7]. Some of the deoxynucleoside requiring lactobacilli owe their auxotrophy to the requirement of their nucleotide reductases for vitamin B_{12}. Since milk is a fine source of B_{12} these lactobacilli should thus not require deoxy-compounds in milk.

Many prototrophic lactic acid bacteria are apparently stimulated by addition of purines to the growth medium (F. Bringel and J. Martinussen, unpublished). Addition of purines to *L. lactis* MG1363 growing in chemically defined GSA medium stimulated the growth rate by approximately 15% while pyrimidines had no effect [8]. When, however, a good peptide source as casitone was added in combination with purines, the cells grew considerably faster. Under these conditions the growth rate appears to be determined by the capacity of the pyrimidine de novo pathway because uridine could now further stimulate growth (C. Solem and P. Ruhdal Jensen, unpublished). It thus appears that *L. lactis*, and possibly other lactic acid bacteria has a limited capacity for de novo synthesis of both purines and pyrimidines, which is barely enough to support growth in milk. A pyrimidine source is present in milk in the form of orotate, an intermediate of the pyrimidine biosynthesis, but only some strains of lactic acid bacteria can utilize orotate, and orotate will therefore not necessarily support growth of all auxotrophic strains.

2. 5-Phosphoribosyl-α-1-pyrophosphate synthesis

A common intermediate in both purine and pyrimidine metabolism is 5-phosphoribosyl-α-1-pyrophosphate (PRPP). It is used in both the biosynthesis of nucleotides and the salvage of nucleobases. PRPP is also required for the biosynthesis of nicotinamide coenzymes (NAD^+ and $NADP^+$) and for the amino acids histidine and tryptophane (Fig. 1). As discussed below, *L. lactis* and most other lactic acid bacteria rely on phosphoribosylation of bases in purine salvage because they cannot directly phosphorylate purine ribonucleosides (Fig. 2). Consequently, PRPP is essential in these organisms. It has been shown that apart from its role as a substrate in biosynthetic pathways, PRPP is an inducer of the purine de novo gene expression in *L. lactis*. Expression of the purine biosynthetic genes is dependent on an activator, PurR, which stimulates transcription under high PRPP concentrations [9]. The de novo pathway is thus feed-forward regulated by its precursor, PRPP, to ensure that the pathway is only fully active when the substrate is found in excess. As explained later, the PRPP concentration is also modulated by the salvage of purines, so it is an indicator of the purine availability.

PRPP is formed from ribose 5-phosphate and ATP by the PRPP synthase (EC 2.7.6.1). In the reaction, the pyrophosphate group from ATP is transferred to the 1 position of ribose 5-phosphate. The enzyme has been purified and characterized from a number of different organisms including *Escherichia coli* [10] and *Bacillus subtilis* [11]. A common feature of the characterized enzymes is that ADP inhibits the catalytic activity. The three-dimensional structure of PRPP synthase from *B. subtilis* has been determined with and without ligands, and by combining the results from kinetic studies with the conformational changes in the enzyme upon binding of ligands, detailed reaction models for the catalytic function and allosteric regulation have been proposed [12].

E. coli and *B. subtilis* carry one gene encoding a PRPP synthase, but surprisingly, two different open reading frames *prsA* and *prsB*, with a high degree of similarity to known PRPP synthase genes could be

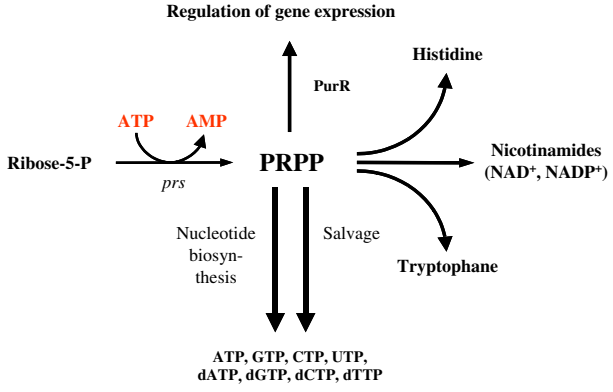

Fig. 1. 5-Phosphoribosyl-α-1-pyrophosphate (PRPP) synthesis and utilization. PRPP is synthesized by addition of a pyrophosphate group from ATP to ribose-5-phosphate. The reaction is catalyzed by the PRPP synthase, encoded by the *prsA* and *prsB* genes. PRPP is utilized in both biosynthesis and salvage of purines and pyrimidines, biosynthesis of the amino acids histidine and tryptophane, and production of nicotinamides used as prosthetic groups in a number of different enzymes. Finally, PRPP is an effector molecule in PurR-mediated gene regulation.

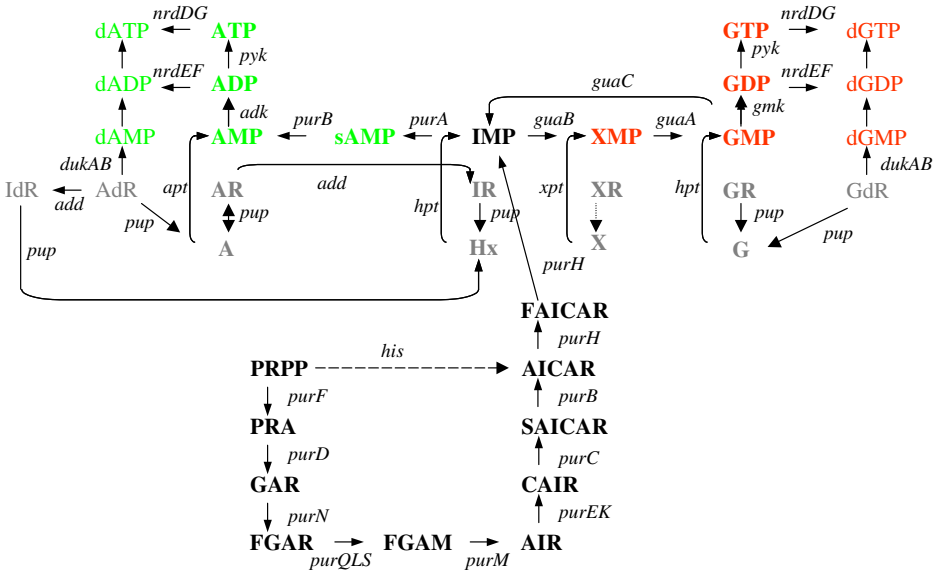

Fig. 2. The pathways of purine biosynthesis and salvage in *L. lactis*. The individual enzymes are presented by their gene symbols. Arrows indicate the direction of reactions observed in vivo in wild-type cells grown in defined medium. The following abbreviations are used: PRPP, Phosphoribosyl pyrophosphate; PRA, phosphoribosyl amine; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, aminoimidazole ribonucleotide; CAIR, phosphoribosyl carboxyaminoimidazole; SAICAR, succinocarboxyamide carboxyaminoimidazole ribonucleotide; AICAR, aminoimidazole carboxamide ribonucleotide; FAICAR, formaminoimidazole carboxamide ribonucleotide; A, adenine; Hx, hypoxanthine; X, xanthine; G, guanine; IdR, deoxyinosine; AdR, deoxyadenosine; AR, adenosine; IR, inosine; XR, xanthosine; GR, guanosine; GdR, dexyguanosine; sAMP, adenylsuccinate; IMP, inosine monophosphate; XMP, xanthosine monophosphate. Enzymes encoded by the genes in alphabetical order, *add*, adenosine deaminase; *adk*, adenylate kinase (AMP kinase); *apt*, adenine phosphoribosyltransferase; *dukAB*, deoxynucleoside kinase; *gmk*, guanylate kinase (GMP kinase); *guaA*, GMP synthase; *guaB*, IMP dehydrogenase; *guaC*, GMP reductase; *hpt*, hypoxanthine/guanine phosphoribosyltransferase; *nrdDG*, anaerobic ribonucleotide triphosphate reductase; *nrdEF*, aerobic ribonucleotide diphosphate reductase; *pup*, purine nucleoside phosphorylase; *purA*, adenylosuccinate synthase; *purB*, adenylosuccinate lyase; *purC*, SAICAR synthase; *purD*, GAR synthase; *purEK*, CAIR synthase; *purF*, PRPP amidotransferase; *purH*, bifunctional AICAR transformylase/IMP cyclohydrolase; *purM*, AIR synthase; *purN*, GAR transformylase; *purQSL*, FGAM synthase; *pyk*, pyruvate kinase; and *xpt*, xanthine phosphoribosyltransferase.

identified in the genome sequence of *L. lactis* subsp. *lactis* IL1403 [13]. These genes are also found in *L. lactis* MG1363, by PCR analysis and sequencing (Jan Martinussen, unpublished). DNA array analysis has furthermore shown that both of these genes are transcribed in *L. lactis* MG1363 (Mogens Kilstrup, unpublished), and both PrsA and PrsB have accordingly been identified in a proteomic analysis of *L. lactis* [1]. A search in the genomic sequences of *Lactobacillus plantarum* and *Lactobacillus johnsonii* suggested that these species likewise harbor two *prs* alleles, but the *S. thermophilus* genome sequence only showed one *prs* gene (data not shown). The possible role of two separate *prs* genes is unknown.

3. Pyrimidine de novo synthesis

3.1. Synthesis of dihydroorotate

The end product of the pyrimidine biosynthetic pathway is UMP (Fig. 3). It is formed through six enzymatic steps utilizing carbon dioxide, ammonia, ATP, aspartate, PRPP, and phosphate. With one exception, the properties of the enzymes are not well characterized in lactic acid bacteria. The forth step in pyrimidine biosynthesis is the reduction of dihydroorotate to orotic acid catalyzed by dihydroorotate dehydrogenase. The reaction and the enzyme have been studied in great detail in *L. lactis*. Therefore, the current knowledge of that reaction will be reviewed below. The other reactions will be briefly presented.

The first step in the pyrimidine biosynthetic pathway is the formation of carbamoyl phosphate (CP) (Fig. 3). This compound is precursor in the biosynthesis of both pyrimidines and the amino acid arginine. One molecule of CP is formed by fusing bicarbonate with an ammonia group from asparagine, and a phosphate group obtained from ATP. A second ATP molecule is hydrolyzed in order to donate sufficient energy to drive the reaction. The formation of CP is catalyzed by Carbamoylphosphate Synthase (EC 6.3.5.5). The heteromeric enzyme from *L. lactis* consists of a 118 kDa synthase subunit [14] and a 40 kDa glutaminase subunit [15]. In contrast to other organisms, the genes encoding the two subunits, CarA and CarB are not physically clustered. The large subunit is transcribed from *carB* as a monocistronic message [14], whereas the *carA* gene encoding the

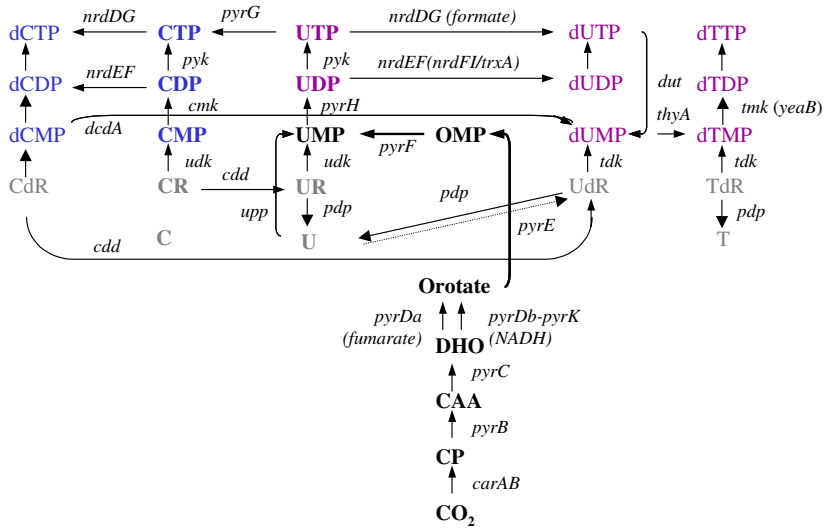

Fig. 3. The pathways of pyrimidine biosynthesis and salvage in *L. lactis*. The individual enzymes are presented by their gene symbols. Arrows indicate direction of reactions observed in vivo in wild-type cells grown in defined medium. The following abbreviations are used: CP, carbamoylphosphate; CAA, carbamoylaspartate; DHO, dihydroorotate; OMP, orotate monophosphate; C, cytosine; U, uracil; T, thymine; CR, cytidine; UR, uridine; CdR, deoxycytidine; UdR, deoxyuridine; and TdR, thymidine. Enzymes encoded by the genes in alphabetic order: *carAB*, carbamoylphosphate synthase; *cdd*, cytidine deaminase; *cmk*, CMP kinase; *dcdA*, dCMP deaminase; *dut*, dUTPase; *nrdDG*, anaerobic ribonucleotide triphosphate reductase; *nrdEF*, aerobic ribonucleotide diphosphate reductase; *pdp*, pyrimidine nucleoside phosphorylases; *pyk*, pyruvate kinase; *pyrB*, aspartate transcarbamoylase; *pyrC*, dihydroorotase; *pyrDa*, dihydroorotate dehydrogenase A; *pyrDb*/*pyrK*, dihydroorotate dehydrogenase B; *pyrE*, orotate phosphoribosyltransferase; *pyrF*, OMP decarboxylase; *pyrG*, CTP synthase; *pyrH*, UMP kinase; *tdk*, thymidine kinase; *thyA*, thymidylate synthase; *tmk*, (d)TMP kinase; *udk*, uridine kinase; and *upp*, uracil phosphoribosyltransferase.

glutaminase subunit is the last gene in the *pyrRPBcarA* operon [15] (Fig. 4). Despite the fact that CP is required for both pyrimidine and arginine biosynthesis, the *carA* and *carB* genes are only regulated by pyrimidine availability in *L. lactis* [14]. In *Lb. plantarum* two different enzymes catalyzing carbamoyl phosphate synthesis is present (Fig. 4). One set of genes (*pyrAa1Ab1*) is member of the pyrimidine biosynthetic operon, and as such regulated by pyrimidine availability (see below) [16]. The *carAB* operon encoding the other enzyme, is member of the arginine regulon [17]. The CPSase encoded by the pyrimidine operon produced enough CP for both pathways, but only in the absence of exogenous uracil, which repressed gene expression. Moreover, prototrophic *Lb. plantarum* was unable to grow without CO_2 enrichment in the presence of uracil. It may be due to a low affinity of the arginine-regulated CPSase for its substrate CO_2 or to regulation of the CP pool by the cellular bicarbonate level [18].

The second step, in which carbamoylphosphate is reacting with aspartate to yield carbamoylaspartate, is catalyzed by aspartate transcarbamoylase (ATCase, EC 2.1.3.2). In *B. subtilis* the ATCase has been shown to be a trimer of identical polypeptides [19]. Since the *L. lactis* MG1363 ATCase is closely related to the *B. subtilis* enzyme [15], it is reasonable to believe, that also the *L. lactis* enzyme is a homo-trimer.

The dihydrorotase (DHOase, EC 3.5.2.3) catalyzes the formation of the first ring structure by subjecting carbamoylaspartate to an intramolecular amide formation, yielding dihydroorotate. This reaction is reversible.

3.2. Dihydroorotate dehydrogenase

In the fourth reaction, the double bond is introduced into the ring, forming the first pyrimidine compound. The reaction is catalyzed by dihydroorotate dehydrogenases (EC 1.3.3.1) (Fig. 3). The PyrD reactions and the corresponding enzymes from *L. lactis* are very well studied. Because of their thorough investigation, the lactococcal enzymes are considered the model enzymes of dihydroorotate dehydrogenases in gram-positive organisms. Surprisingly, two different enzymes have been found to catalyze the conversion of DHO to orotate in *L. lactis*, with the use of different electron donors. PyrDa encoded by *pyrDa* was shown to be dependent on fumarate, whereas PyrDb encoded by *pyrDb* makes use of NAD^+ as electron acceptor [20]. A genetic analysis revealed that the PyrDb enzyme required an additional subunit. This subunit, PyrK, was expressed from the *pyrK* gene in the *pyrKDbF* operon [21]. Purification of the enzyme confirmed the genetic evidence, and it could be concluded that the active enzyme is a complex between the PyrDb and PyrK subunits [22]. The PyrDb subunit was extremely unstable in the absence of PyrK. Moreover, the oxidation of dihydroorotate with NAD^+ as electron acceptor was dependent on the PyrK subunit [22]. A *pyrK*, *pyrDa* double mutant was found to have a

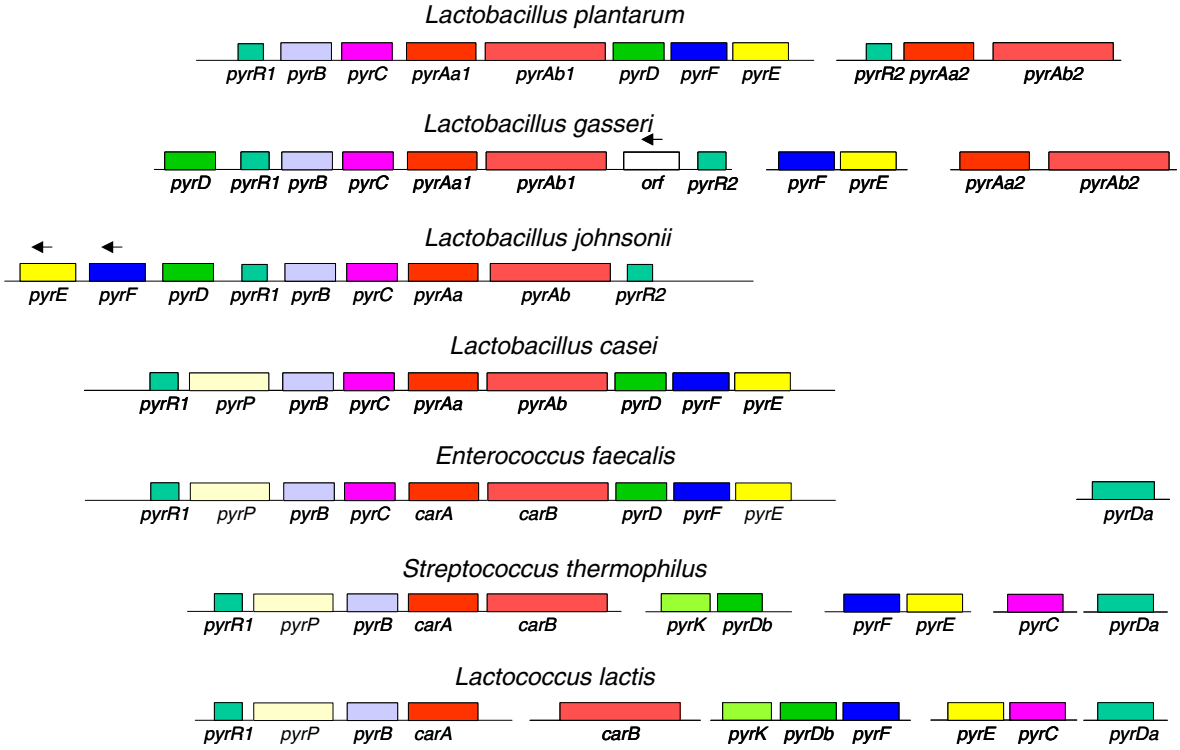

Fig. 4. Organization of the genes for the pyrimidine de novo synthesis in lactic acid bacteria. The following organisms are included: *Lb. plantarum* CCM1904, *Lb. gasseri* ATCC 33323, *Lb. johnsonii* NCC 533, *Lb. casei* ATCC 334, *E. faecalis* OG1RF, *S. thermophilus* CNRZ1066, and *L. lactis* subsp. *cremoris* MG1363. The genes encoding the following proteins are shown: *pyrR*, the pyrimidine regulatory protein PyrR; *pyrAa*, pyrimidine specific carbamoylphosphate synthase small subunit; *pyrAb*, pyrimidine specific carbamoylphosphate synthase large subunit; *carA*, carbamoylphosphate synthase small subunit; *carB*, carbamoylphosphate synthase large subunit; *pyrB*, aspartate transcarbamoylase; *pyrC*, dihydrorotase; *pyrDa*, dihydroorotate dehydrogenase A; *pyrDb*, dihydroorotate dehydrogenase B; *pyrE*, orotate phosphoribosyltransferase; *pyrF*, OMP decarboxylase; and *pyrP*, uracil permease. Gene names followed by the numbers 1 and 2 indicate that two different alleles encoding proteins with similar function are present in the organism. All genes are transcribed from left to right, except when indicated otherwise by an arrow.

partial pyrimidine requirement, suggesting that the PyrDb subunit is partially functional in vivo in the absence of PyrK [21]. The crystal structure of PyrDb/PyrK enzyme has been solved, showing that the enzyme is a hetero-tetramer containing two subunits of PyrDb and two subunits of PyrK. In addition, the PyrDb subunits contain two molecules of flavin mononucleotide (FMN), two molecules of flavin adenine dinucleotide (FAD), as well as two iron–sulfur clusters as tightly bound cofactors [23]. Based on a refinement of the structure, it was possible to deduce the binding sites for dihydroorotate and NAD, and to propose the amino acid residues that constitute the electron transfer pathway from NAD to dihydroorotate in the enzyme [24]. Recently, a detailed thermodynamic framework for electron transfer in PyrDb/PyrK has been established, based on determination of the reduction potentials of the cofactors bound to the mature protein [25].

The PyrDa enzyme is less complex. It is a homodimer of 34 kDa PyrDa subunits that each contain one molecule of flavin mononucleotide (FMN) [26,27]. The most important residues in the active site involved in interactions with dihydroorotate were identified by chemical modification, site-directed mutagenesis, and crystallographic studies of the enzyme containing orotate in the active site [28,29]. It was shown that PyrDa is only functional as a dimer, which dissociates when present in dilute solutions, and at low salt concentrations [30]. This was an interesting observation because it was known that the membrane bound *E. coli* PyrD is monomeric [31]. Recently, the structure of the native enzyme was further refined, adding to the description of enzymatic structure/function relationships among PyrD enzymes [32].

The presence of two fully functional DHODases with different electron donors is unusual, and the reason for this is not understood yet. Strains with single mutations in *pyrDa* and *pyrDb* have been constructed, and their phenotypes have been analyzed in the absence and presence of oxygen. No significant effects of the mutations were found (Els Defoor and Jan Martinussen unpublished). It could be speculated that the two PyrD enzymes may work in opposite directions, enabling a way by which reduction equivalents from NADH may

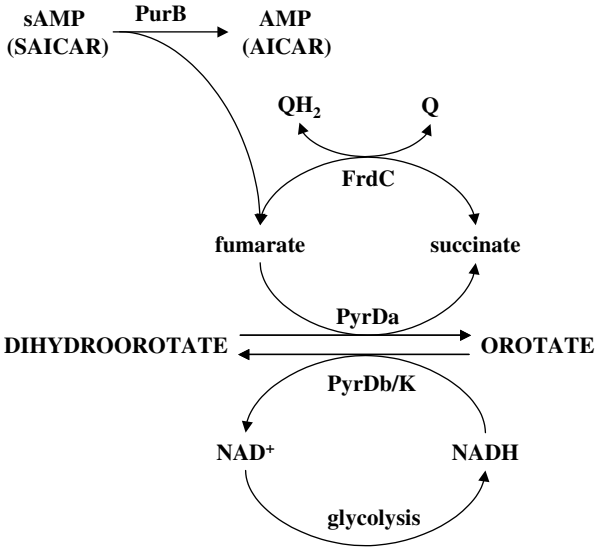

Fig. 5. Reactions linking the purine and pyrimidine de novo pathways to glycolysis and redox potential in *L. lactis*. Fumarate is formed in two steps in the biosynthetic pathway leading to AMP (Fig. 2), both catalyzed by PurB; when SAICAR is converted to AICAR and when sAMP is converted to AMP. Fumarate can be used in the conversion of dihydroorotate to orotate catalyzed by PyrDa, thus linking the purine and pyrimidine de novo pathways. The two PyrD enzymes could theoretically function as a shuttle of electrons between the NADH and quinone pools by the aid of FrdC, the fumarate reductase.

be shunted to the quinone pool during conditions where the NADH oxidase activity might be impaired (Fig. 5). It is also interesting to note that fumarate is produced in two of the reactions in the biosynthesis of AMP (see below), so the use of fumarate as electron acceptor for the PyrDa reaction could serve as a link between the purine and pyrimidine biosynthesis.

3.3. Formation of UMP

Orotic acid is converted to UMP in step five and six in the pyrimidine biosynthesis. The fifth step is the formation of the first pyrimidine nucleotide. Orotate is phosphoribosylated to OMP by PRPP with concomitant pyrophosphate formation (Fig. 3). Subsequently, pyrophosphate is hydrolyzed in order to drive the reaction towards OMP formation, which means that OMP formation is physiologically irreversible. The reaction is catalyzed by orotate phosphoribosyltransferase (OPRTase, EC 2.2.4.10), encoded by *pyrE*. The *pyrE* gene is the first gene in the bicistronic *pyrEC* operon found in both *L. lactis* subspecies (J. Martinussen, unpublished), [13].

The sixth and last step in UMP formation is catalyzed by OMP decarboxylase (EC 4.1.1.23) (Fig. 3). The reaction is irreversible as the decarboxylation is energetically highly favorable. As mentioned above, the *pyrF* gene encoding the OMP decarboxylase is part of the *pyrKDbF* operon.

3.4. Organization and regulation of pyrimidine biosynthetic genes in L. lactis

The pyrimidine biosynthetic genes are scattered on the chromosome of *L. lactis* subsp. *cremoris* MG1363. They are organized in five transcriptional units: *carB* [14], *pyrRPBcarA* [15], *pyrEC* (J. Martinussen, 2000, unpublished), *pyrKDbF* [21] and *pyrDa* [20] (Fig. 4). The same orginazation was found in *L. lactis* subsp. *lactis* IL1403 [13]. The first gene of the *pyrRPBcarA* operon is *pyrR* encoding a regulatory protein. Mutational inactivation of *pyrR* results in a 3- to 8-fold derepression in the expression of all pyrimidine biosynthetic genes except *pyrDa* [15]. The members of the PyrR regulon are regulated through an attenuator mechanism [15]. The same mechanism is believed to be widespread among gram-positive bacteria, including all lactic acid bacteria studied so far, since *pyrR* homologs have been identified in the genome sequences. The pyrimidine attenuator and the PyrR regulatory gene have been studied in great detail in *B. subtilis* (for a review see [33]). A model is presented in Fig. 4. The 5′ leader mRNA of the pyrimidine biosynthetic genes can be folded into two, mutually exclusive structures, one of which is stabilized by the PyrR protein. Without PyrR stabilization the mRNA forms an antiterminator structure (II). In the presence of UMP-bound PyrR, an anti-antiterminator structure (I) is formed, preventing the formation of the antiterminator (II), and thereby permitting the formation of a terminator structure (III). The trailing strand of the antiterminator overlaps the 5′ strand of the terminator (III), so that formation of the terminator is prevented. The anti-antiterminator (I) hairpin is formed by an alternative folding including part of the antiterminator, thereby preventing base pairing in the antiterminator stem (II). Although the leader structures in other bacteria are different with respect to the primary sequence, the functionality is preserved. A stretch of bases overlapping the anti-antiterminator is preserved. This motif has been identified as the PyrR binding site (Fig. 6). The PyrR regulatory protein shows similarity with uracil phosphoribosyl transferases (Upp), catalyzing the conversion of uracil to UMP. Indeed the *B. subtilis* PyrR was shown to possess a significant enzymatic activity, both in vitro and in vivo [34]. In contrast, no Upp activity of PyrR could be identified in *L. lactis* [15]. The PyrR protein combines with UMP, and binds, in this conformation, to the PyrR binding site on the 5′ leader mRNA, thereby stabilizing the anti-antiterminator and the terminator. This leads to low levels of the pyrimidine biosynthetic enzymes.

Based on a proteomic analysis of *L. lactis* NCDO763, the expression of the pyrimidine biosynthetic genes was repressed upon growth in rich M17 medium supplied with lactose, compared to growth in medium supplemented with glucose [1]. The rationale for this regulation

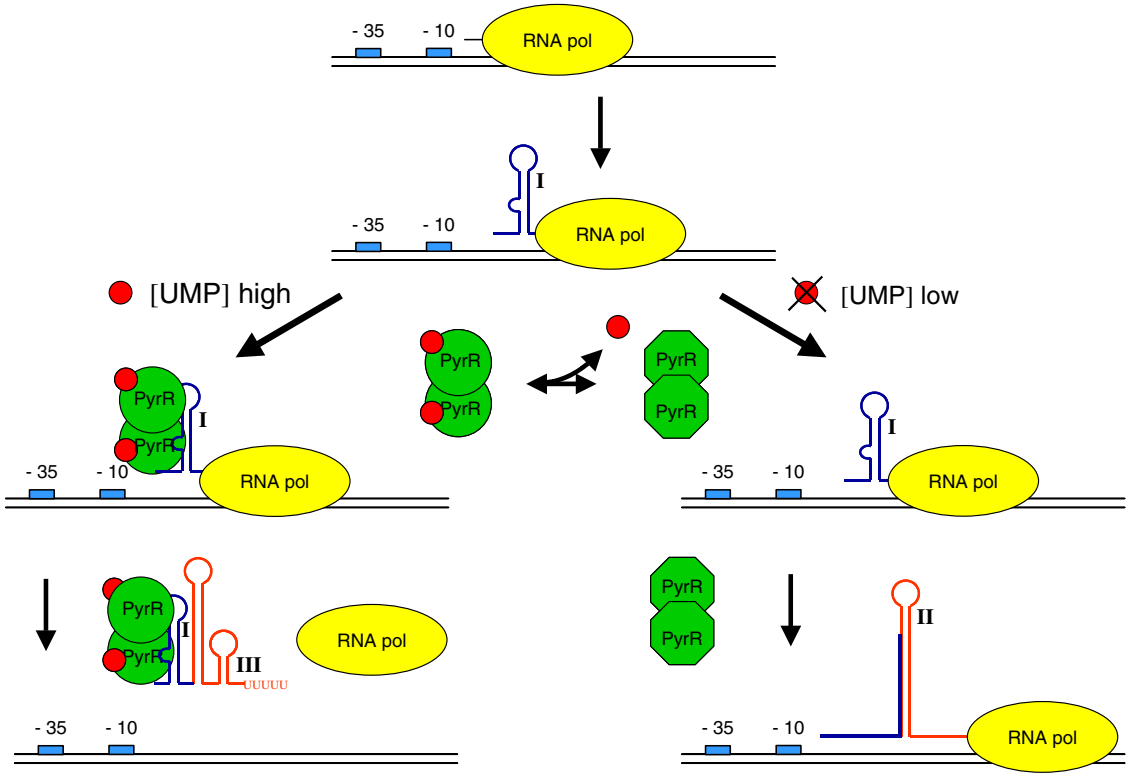

Fig. 6. Model for PyrR-mediated regulation. The promoters of the pyrimidine biosynthetic genes constitutively initiate transcription, and structure (I) is formed. The PyrR protein senses the UMP level in the cell: when pyrimidines are abundant, the UMP concentration is high, facilitating formation of the PyrR–UMP complex. This complex has the capability to bind to a specific sequence in the anti-antiterminator (structure I), thereby stabilizing it. Consequently, the formation of the anti-terminator (II) is prevented, favoring the formation of the terminator (III). RNA polymerase will preferentially terminate. At low UMP level, PyrR is unable to bind to the anti-antiterminator and the energetically more favorable anti-terminator is formed. The terminator cannot form and transcription will proceed beyond the terminator. The structural genes are expressed to a higher degree and more UMP is synthesized. Red circles represent UMP, the green circles represent PyrR in its RNA binding conformation. Green octagons represent PyrR unable to recognize the anti-antiterminator. −35 and −10 indicates the constitutive promoter.

is not clear, but it indicates that the UMP levels are elevated in the presence of lactose. This is an example of the close interdependence between nucleotide synthesis and other metabolic pathways. Also lack of thioredoxin reductase (*trxB1*) was shown to induce PyrE in *L. lactis* MG1363 [35].

3.5. Pyrimidine biosynthesis genes in other lactic acid bacteria

The genes encoding the pyrimidine biosynthesis pathway in *Lb. plantarum* CCM 1904 have been sequenced [16]. They are organized in a single operon (Fig. 4), and very similar to the corresponding operon in *B. subtilis* [36]. There are, however, two major differences when comparing the pyrimidine operons of *B. subtilis* and *Lb. plantarum*: the absence in *Lb. plantarum* of *pyrP* encoding the uracil permease, and the absence of the gene encoding the electron transfer subunit in the dihydroorotate dehydrogenase *pyrDII* (*pyrK* in *L. lactis*). An identical organization was seen in *Lb. plantarum* WCFS1 [37]. Searching for a *pyrK* homolog in *Lb. plantarum* was unsuccessful, suggesting that the *Lb. plantarum* PyrD is a member of yet another PyrD family. Sequence analysis of the 5′ leader region of the *Lb. plantarum pyr* operon suggested that the *pyr* operon in this organism may be regulated by transcription attenuation at two possible attenuation sites. The first attenuation site is located between the promoter and *pyrR1*. The second attenuator is located between *pyrR1* and *pyrB*. The second attenuator has been shown in a recent paper to be the major regulatory site in vivo. Moreover, a second *pyrR* allele (*pyrR2*) was identified as the first gene in a small operon constituted by *pyrAa2PyrAb2* and *pyrR2* [38]. No functional enzyme is expected to be produced from the pseudogene *pyrAb2* due to frameshift mutations [38]. In the same paper it was shown that in a *pyrR1* deletion mutant, the expression of the pyrimidine operon was only partially derepressed, suggesting that also PyrR2 is involved in regulating expression of the pyrimidine biosynthetic genes.

The genes relating to pyrimidine biosynthesis are differently organized in the genomes of those lactic acid bacteria of which the full genome sequences are available (Fig. 6). A common feature is the presence of the pyrimidine regulatory protein PyrR. Consequently,

PyrR type attenuators are found upstream of all pyrimidine biosynthetic genes. Moreover, in most lactobacilli two different *pyrR* alleles can be identified. The only exception seems to be *Lb. casei*, but it should be emphasized that the analysis is based on the unfinished genome sequence.

Of special interest is the presence or absence of the *pyrK* allele in different lactic acid bacteria. As discussed above, *pyrK* encodes a subunit of the PyrDb/PyrK enzyme. This subunit is required when NADH is used as electron donor in *L. lactis* (Fig. 5). A *pyrK* allele was found also in *S. thermophilus* and *Enterococcus faecalis*, whereas it was absent in the lactobacilli and in *Oenococcus oeni*. The PyrD enzyme in *O. oeni* is closely related to PyrDa of *L. lactis*, using fumarate as electron donor, whereas the PyrDs of the lactobacilli are very similar to *L. lactis* PyrDb, which uses NAD^+ as electron acceptor. Since the latter type of enzyme is dependent on PyrK (or PyrDII) in both *L. lactis* [21] and *B. subtilis* [39], it was surprising that no *pyrK* homolog could be identified in the genome sequences of lactobacilli. A cluster analysis of the amino acid sequences of all PyrDb enzymes from lactic acid bacteria reveals that the *Lactobacillus* PyrDb enzymes form a separate branch, separated from *L. lactis*, *S. thermophilus*, *E. faecalis*, and *B. subtilis* (Fig. 7). It could be speculated that the dihydroorotate dehydrogenases of lactobacilli use electron acceptors other than NAD^+ and fumarate. The dihydroorotate dehydrogenase from *Lactobacillus bulgaricus* has previously been purified and a partial characterization performed [40,41]. The size of the active enzyme was found to be 53 kDa suggesting it being a dimer. The purified enzyme was analyzed only on native polyacrylamide gels. Therefore, no clues with respect to whether the enzyme is a homodimer or heterodimer could be obtained. The physiological electron donor was not identified in the study.

4. Pyrimidine nucleotide interconversion leading to UTP and CTP

Through the nucleotide interconversion pathways all pyrimidine nucleotides can be produced from UMP, which is first phosphorylated to UDP by the UMP kinase encoded by *pyrH* [42], and subsequently converted to UTP by an NDP kinase, as discussed in the purine interconversion section.

4.1. Formation of CTP

UTP can both be considered as an end product in the nucleotide metabolism and as the precursor of the CTP biosynthetic pathway. The conversion of UTP to CTP is

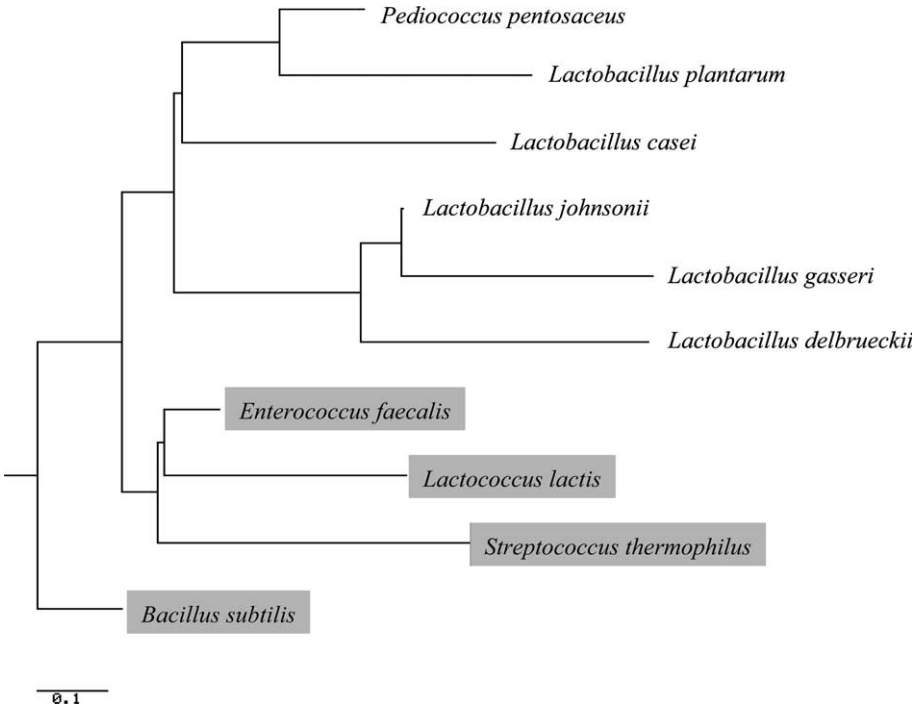

Fig. 7. The phylogenetic tree of dihydroorotate dehydrogenase B enzymes (PyrDb) from different lactic acid bacteria and *B. subtilis*. The construction is based on the cluster algorithm and performed at the TreeTop on-line service found at http://www.genebee.msu.su/services/phtree_reduced.html. The organisms in which a *pyrK* gene has been identified are indicated by shading. The following organisms are included: *Pediococcus pentosaceus* ATCC 25745, *Lb. plantarum* CCM1904, *Lb. casei* ATCC 334, *Lb. johnsonii* NCC 533, *Lb. gasseri* ATCC 33323, *Lb. delbrueckii* ATCC BAA-365, *E. faecalis* OG1RF, *L. lactis* subsp. *cremoris* MG1363, *S. thermophilus* CNRZ1066, and *B. subtilis* 168.

catalyzed by PyrG/CTP synthase (EC 6.3.4.2) encoded by *pyrG*. *L. lactis* strains harboring disrupted *pyrG* alleles required cytidine for growth, showing that PyrG is the only enzyme responsible for the amination of UTP to CTP in *L. lactis* [43]. CTP synthase catalyzes the transfer of an amino group to the pyrimidine ring in UTP to yield CTP. The reaction proceeds by activation of the base moiety of UTP by ATP-dependent phosphorylation. The activated intermediate reacts with an ammonia molecule obtained by hydrolysis of glutamine.

An analysis of *L. lactis* PyrG revealed differences compared to PyrG enzymes from other organisms. Unlike the majority of CTP synthases the lactococcal enzyme converts dUTP to dCTP, although low affinity towards dUTP makes the physiological role questionable. The structure of the lactococcal enzyme was found to be a tetramer, but unlike most of the other previously characterized PyrG enzymes, the tetramer was very stable even at dilute enzyme concentration [43]. The rate of CTP formation by PyrG is greatly enhanced by the allosteric activator GTP. Normally GTP activation is believed to be tightly coupled to the glutaminase half-reaction, but for *L. lactis* PyrG, the binding of GTP to the allosteric site increases the coordination of the phosphorylation of UTP with the hydrolysis of glutamine for optimal efficiency in CTP formation [44,45]. The residues important for GTP binding have been identified in a mutational study [46]. In addition to glutamine, which is hydrolyzed by the enzyme to ammonia and glutamate, CTP synthase will also utilize externally added NH_4Cl. This reaction is not dependent on allosteric activation by GTP [47].

4.2. Regulation of pyrG expression

The expression of *pyrG* of *L. lactis* is regulated at the level of transcription through an attenuator mechanism. Upon CTP starvation the *pyrG* expression is increased 40-fold [48]. Moreover, the expression of *pyrG* was found to respond to intracellular CTP concentration. A model (Fig. 8) for the regulatory mechanism introducing a protein independent attenuator control was proposed by data obtained from a comparison of sequences from different *pyrG* leaders [48], and it was further refined by data from the *pyrG* gene in *B. subtilis* [49].

Transcription is initiated with a 5′-GGGCACTGG sequence, which is transcribed in the presence of excess CTP. Further downstream a region of dyad symmetry followed by a stretch of U residues forming a transcriptional terminator can be identified. During CTP deficiency the RNA polymerase pauses immediately upstream of the C residue at position four, causing reiterative transcription or stuttering. As a result additional G residues are incorporated in the extreme 5′ end of the RNA. An antiterminator can be formed only after reiterative initiation (Fig. 8). When the antiterminator is formed, transcription proceeds, resulting in expression of *pyrG*. Sequence analyses suggest that many different organisms including lactic acid bacteria use the same mechanism.

4.3. Control analysis of PyrG

Metabolic control analysis is a mathematical tool used to describe to which extent a given enzyme and its corresponding gene in a metabolic pathway control fluxes and metabolite concentrations. This technology was used to address the question whether CTP synthase encoded by *pyrG* controls CTP formation. A library of strains with altered *pyrG* expression from 3% to 665% of the wild-type level was constructed by inserting synthetic promoters upstream of *pyrG*. Decreasing the level of CTP synthase to 43% had no effect on the growth rate, showing that there is excess capacity of CTP synthase in the wild-type cells [50]. In contrast, *pyrG* expression had a very strong effect on the intracellular pool sizes of CTP, UTP, and dCTP. The correlation between *pyrG* expression and nucleotide pool sizes was quantified in terms of inherent control coefficients using metabolic control analysis [51]. At the wild-type expression level, CTP synthase had full control of the CTP concentration with a concentration control coefficient close to one and a negative concentration control coefficient of −0.28 for the UTP concentration. The concentration control coefficient for the dCTP concentration was calculated to be 0.49 [50]. Replacing the normal *pyrG* promoter and leader region with a constitutive promoter eliminated any regulation that might have occurred at the transcriptional level. Indeed, since strong regulation of *pyrG* expression is known to take place at the transcriptional level in response to changes in the intracellular CTP pool, such regulation will almost eliminate the observed control. These results emphasize how important it is to take genetic regulation into account when building in silico models of metabolic processes.

5. Formation of deoxyribonucleotides

5.1. The ribonucleotide reductases (Nrd)

Nucleotides can be reduced to the corresponding deoxy-nucleotides at either the NDP or NTP level depending on the type of nucleotide reductase (Nrd) present in the bacterium and the growth conditions. Three classes of Nrd enzymes have been found in nature, all of them have U, A, G, and C nucleotides as substrates but the substrate specificities of the enzymes are under allosteric control [52,53]. The catalytic activity of all Nrd enzymes is dependent on radical formation.

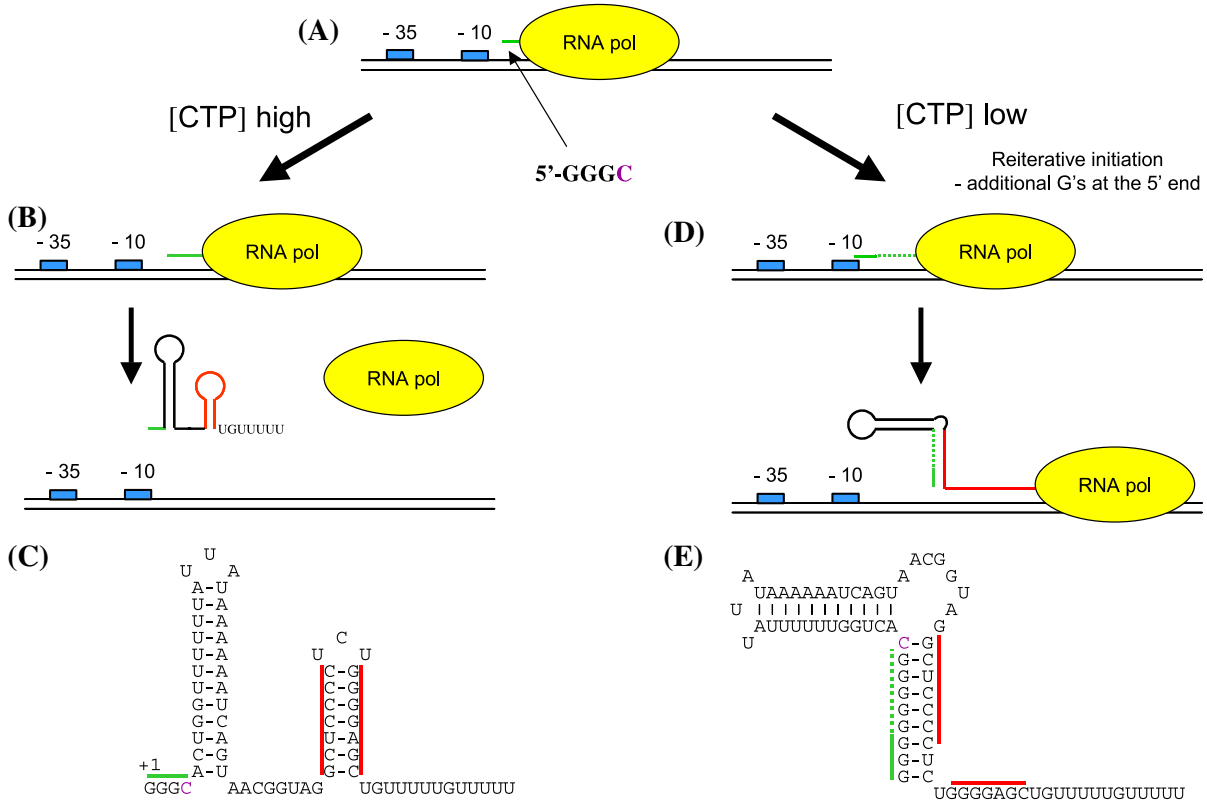

Fig. 8. Model for *pyrG* regulation. (A) Transcription is initiated with a 5′-GGGC sequence (green line). (B) During high CTP concentration, transcription proceeds and a terminator involving base-pairing in the region indicated by red is preferentially formed. (C) The secondary structure of the *pyrG* leader in the terminator configuration. (D) At CTP shortage the RNA polymerase pauses immediately in front of the purple C residue at position four in the 5′-GGGC sequence. Due to reiterative transcription or stuttering, additional G residues are incorporated in the extreme 5′ end of the RNA, indicated by a stippled green line. This stretch of G residues (green line) pairs with the left-hand side of the terminator stem (red line), thereby preventing formation of the terminator. Transcription proceeds resulting in expression of the *pyrG* open reading frame. (E) The secondary structure of the *pyrG* leader in the antiterminator configuration.

In lactic acid bacteria all three classes of Nrd enzymes are found [52,54]. Nrd class I enzymes (NrdEF) utilize a radical, generated on a tyrosine residue in the active site, which requires oxygen. Nrd I enzymes utilize NDPs as substrates. Nrd class II enzymes utilize a radical formed on the cofactor adenosylcobalamine (coenzyme B12). These enzymes function independently of the presence of oxygen. The first Class II Nrd discovered was the reductase from *Lactobacillus leishmannii*, which has NTPs as substrates. Later other Class II enzymes having NDP as substrates have been discovered [52]. Nrd class III enzymes utilize a radical generated on a glycyl residue in the active site requiring strictly anaerobic conditions. Nrd III has NTP as substrates and formate as hydrogen donor. Bacteria, which can grow both aerobically and anaerobically, cannot live with only an Nrd I or an Nrd III enzyme. They either contain both enzymes (as *L. lactis* and *E. coli*) or they have a single Nrd II enzyme as *Lb. leishmannii*. Species having only enzyme II are expected to require B_{12} or deoxyribonucleosides, as has been reported also for *Lb. lactis* [55]. Some bacteria have also been found to possess both Nrd I and Nrd II [52].

In *L. lactis* nucleotides can be reduced to the corresponding deoxy-nucleotides at either the NDP or NTP level (Figs 2 and 3). Under aerobic conditions ADP, GDP, CDP, and UDP are reduced to dADP, dGDP, dCDP, and dUDP by the NrdEF nucleotide reductase (class I) [56]. The electron donor in the reaction is the reduced form of the glutaredoxin-like NrdH protein. It is not known which reductase is converting the oxidized NrdH protein back to the reduced form, but in *E. coli*, the homologues NrdH protein utilizes the TrxB thioredoxin reductase [57]. The NrdH protein has been defined as a glutaredoxin-like protein with a thioredoxin-like activity profile [57], due to the presence of a thioredoxin-like domain, which makes it a substrate for thioredoxin reductases. The ultimate electron donor for the reaction is likely to be NADPH, the coenzyme for the TrxB enzyme, and the Nrd I protein appears to be important for the process [57]. All the genes involved in the aerobic nucleotide reductase are clustered and presumably form an *nrdHIEF* operon in *L. lactis* [56], and likewise in *E. faecalis*, *Lb. plantarum*, and *S. thermophilus*, although *nrdI* homologues have not been annotated in the two latter species. In *B. subtilis* the

homologous operon is subjected to induction by thymidine starvation in a RecA-dependent manner [58]. In *L. lactis* the NrdEF enzyme is induced when the deoxynucleotide products are low [54].

Under anaerobic conditions the NrdEF enzyme is rendered inactive because the activation of the catalytic tyrosyl radical requires molecular oxygen. This was earlier a controversial issue because nucleotide reduction was found to be sufficient for growth under near anaerobic conditions in a mutant where the anaerobic reductase was inactivated [56]. It was, however, shown that under strict anaerobic conditions, where all traces of oxygen had been removed by the addition of sulfide, the mutant was unable to produce deoxynucleotides. Under anaerobic conditions the wild-type NrdG protein (Nrd III) becomes active and takes over nucleotide reduction at the triphosphate level [59]. When the NrdG enzyme is activated, i.e., when it harbors a glycyl radical, it can convert NTPs to their corresponding dNTPs, presumably with formate as the electron donor [60]. After approximately 4000 rounds of NTP reductions, the enzyme has to be reactivated by the NrdD protein at the expense of a molecule of reduced flavodoxin and *S*-adenosyl methionine, liberating methionine and 5′deoxyadenosine in the process [59]. A larger class of proteins, including NrdG, which utilize similar oxygen sensitive enzymatic reactions, has been called the Radical SAM protein family. The pyruvate formate lyase is another member of this family. This enzyme is of particular interest in relation to dairy fermentations because it provides acetyl-CoA for the synthesis of fatty acids and aroma compounds under anaerobic conditions [61,62]. Interestingly, an important synergy is likely to exist between these two Radical SAM enzymes because the formate that is produced as a by-product by pyruvate formate lyase, in the conversion of pyruvate to acetyl-CoA, is utilized as the electron donor in nucleotide reduction (Fig. 9). Furthermore, NTP reduction is not the only reaction in which formate production may influence nucleotide synthesis. Formate is utilized by the Fhs enzyme and incorporated into formyl-tetrahydrofolate, which is the C1 donor in the purine biosynthesis pathway (Fig. 9). Accordingly, the *fhs* gene has been found to be a member of the PurR regulon and is induced under general purine starvation [4].

The genes for the anaerobic NTP reductase Type III form an *nrdDG* operon in *L. lactis*, *Lb. plantarum*, and *E. faecalis*. In *S. thermophilus*, two apparently unrelated genes are present between *nrdD* and *nrdG*. No data have yet been published about the regulation of these genes.

5.2. De novo synthesis of dCTP may require RNA turnover

As explained above, the formation of CTP is special compared to the formation of all other NTPs where the NTP is obtained by phosphorylation of the respective NDP by the use of an NDP kinase. The precursor for DNA synthesis, dCTP can be formed by reduction of CTP during anaerobiosis by the ribonucleotide reductase NrdG. This is, however, not always the case. CDP is required for the synthesis of dCDP using the aerobic ribonucleotide reductase, and CDP is therefore an intermediate in the dCTP de novo synthesis pathway under these conditions. In an *E. coli* mutant, it was previously found that a deletion of the *cmk* gene, encoding the CMP kinase (Cmk), resulted in a decreased rate of DNA synthesis [63]. Based upon this and other evidence, the authors suggested that efficient dCTP de novo synthesis required a steady CMP supply from RNA turnover as well as a functional Cmk enzyme to convert CMP to CDP [63]. *E. coli* may possess an alternative route for CDP formation, i.e., the reversion of the nucleotide diphosphate kinase reaction. This reaction is absent in *L. lactis* (see Section 8.2), so CDP formation in this bacterium may require RNA hydrolysis. The entire route from UTP to dCTP under aerobic conditions would thus be: UTP → CTP → RNA → CMP → CDP → dCDP → dCTP. It should be mentioned that the pathway leading to phospholipid synthesis involves a CTP-dependent activation leading to the intermediate CDP-diacylglycerol. Because CMP is cleaved off in the subsequent conversion, this pathway could likewise supply CMP from CTP.

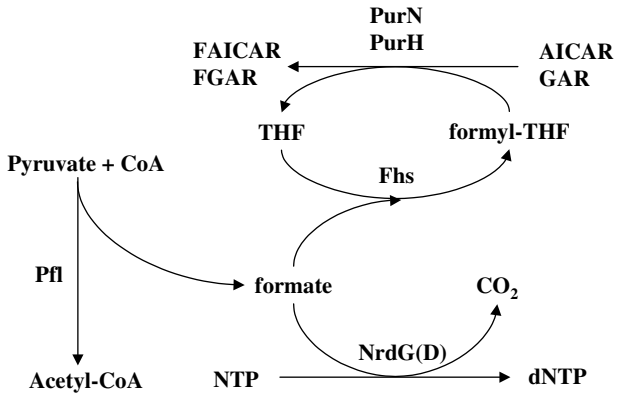

Fig. 9. Formate production and utilization in *L. lactis*. The known pathways for formate metabolism are shown. The enzymes are shown by their genetically derived names as follows: Pfl, pyruvate formate lyase; Fsh, formate-tetrahydrofolate ligase; NrdGD, anaerobic ribonucleosidediphosphate reductase; PurH, AICAR transformylase; PurN, GAR transformylase.

5.3. The formation of dTTP

The key step in the de novo biosynthesis of thymidine tri-phosphate (dTTP) is the methylation step where dUMP is converted to dTMP (Fig. 3). The conversion of dUMP to dTMP is catalyzed by thymidylate synthase (*thyA*), and requires methylene-tetrahydrofolate as

methyl donor. The dUMP required for this reaction is obtained by dephosphorylation of dUTP, in a reaction catalyzed by the *dut*-encoded dUTPase. The *thyA* gene from *L. lactis* has been cloned [64] and the corresponding enzyme was purified and characterized [65]. The first thymidylate synthase three-dimensional structure was obtained from *Lb. casei* [66]. This enzyme has been the subject of a detailed study, and the information has been exploited in the design of inhibitors specific for bacterial thymidylate synthases [67]. 5-Fluoro-dUMP is a potent inhibitor of thymidylate synthase, and acts by forming a stable, covalent bond between the drug and a residue in the active site of the enzyme. The folate analogs trimethoprim and aminopterin have frequently been used to obtain *thyA* mutants, but *L. lactis* is naturally resistant to these drugs [68]. Mutants in *thyA* require thymidine or thymine for growth, as discussed below.

The phosphorylation of dTMP to dTDP is catalyzed by the enzyme thymidylate kinase. The corresponding gene in *L. lactis* is suggested to be *yeaB* due to sequence similarity [13]. Thymidylate kinase solely uses dTMP as substrate, although a residual activity using dUMP as phosphate acceptor has been demonstrated in *S. thermophilus* [69] and yeast [70]. The final phosphorylation to dTTP is catalyzed by a general NDP kinase, as discussed under NTP formation.

6. Purine de novo synthesis

Purine nucleotides are synthesized de novo in a single pathway resulting in the formation of the intermediate IMP. After IMP the path branches into two separate routes, leading to AMP and GMP, respectively.

6.1. Synthesis of IMP

The general outline and the intermediates of the IMP de novo pathway (Fig. 2) are shared by all living organisms that do not require addition of purines. IMP is synthesized in 10 steps from PRPP, by gradual assembly of the purine ring structure on position 1 of the ribose-5-phosphate moiety. Although all intermediates are conserved in living organisms, some steps are catalyzed by enzymes that show variations even among related bacteria. The first reaction is a displacement of the pyrophosphate group of PRPP with the amide group of glutamine, forming PRA. See legend to Fig. 2 for full names of the enzymes and metabolites. The PurF enzyme catalyzing this reaction is conserved in all bacteria [71,72]. The same is the case for the PurD enzyme, which converts PRA to GAR by attaching a glycine moiety to the amine group of PRA. The synthesis of FGAR in *L. lactis* is catalyzed by the PurN enzyme, which transfers a C1 unit from the folate pool [71] to GAR. In *E. coli* and some other bacteria an additional PurT enzyme synthesizes FGAR with formate as the C1 donor [73]. No PurT homologue has so far been detected in the genome sequences of low GC gram-positive bacteria (data not shown). Another point of difference between the bacterial reactions is in the subsequent conversion of FGAR to FGAM. This reaction, which is an ATP-dependent glutamine amidotransferase reaction, requires the PurL protein and, in *B. subtilis*, and most likely also in *L. lactis* and *Lb. plantarum*, two additional subunits, PurQ and PurS [74]. The PurQ subunit is believed to harbor the glutamine amidotransferase domain, and the PurS protein may serve as a hinge between PurQ and PurL [74]. In other bacteria, such as *E. coli* and *S. thermophilus*, the PurQ and PurL enzymes are fused, and the PurS subunit is missing [74]. The genes for both the separate and the fused proteins are designated *purL*. The overall structure of the enzymes of the four reactions from FGAM through AIR (formation of the imidazole ring), CAIR, and SAICAR to AICAR, i.e., PurM, PurEK, PurC, and PurB appear to be conserved in bacteria. The last two reactions in the biosynthesis of IMP are catalyzed by the multifunctional PurH enzyme, which first attaches a C1 unit from the folate pool to AICAR to form FAICAR, and then closes the second ring to form IMP.

Very little is known about the properties of the enzymes from the biosynthetic pathway in gram-positive bacteria. The enzymes of the *B. subtilis* pathway are very similar to those of *L. lactis*. Only PurF, which is the first enzyme in the pathway, has been studied in detail, and its analysis has been very informative. This interesting enzyme is inhibited by AMP and GMP [75], and it is specifically inactivated and degraded in the stationary growth phase in the presence of oxygen [76]. The inactivation may be controlled over a wide range by changing the AMP/GMP ratio because AMP stabilizes the enzyme while GMP destabilizes it [76]. Furthermore it has been shown that PurF is stabilized in a *relA* mutant, suggesting that (p)ppGpp is somehow involved in its degradation [77].

6.2. Genetics of the IMP biosynthetic pathway

Whereas the enzymes of the IMP biosynthesis pathway are very similar in lactic acid bacteria, the organization of the genes encoding the enzymes in the pathway differs (Fig. 10). In *Lb. plantarum* and *E. faecalis* the *pur* genes are organized in a single *purEKCSQLFMNHD* cluster, very similar to the *B. subtilis purEKBCSQLFMNHD* operon [78] except for the omission of the *purB* gene (Fig. 10). The presence of overlapping genes in *Lb. plantarum* [37] and *E. faecalis* [79] *pur* gene clusters strongly suggests that the genes also form an operon in these bacteria. The genes are likewise clustered in a small region on the genome of *L. lactis*, but here the genes are divided into three operons, *purCSQLF* [80],

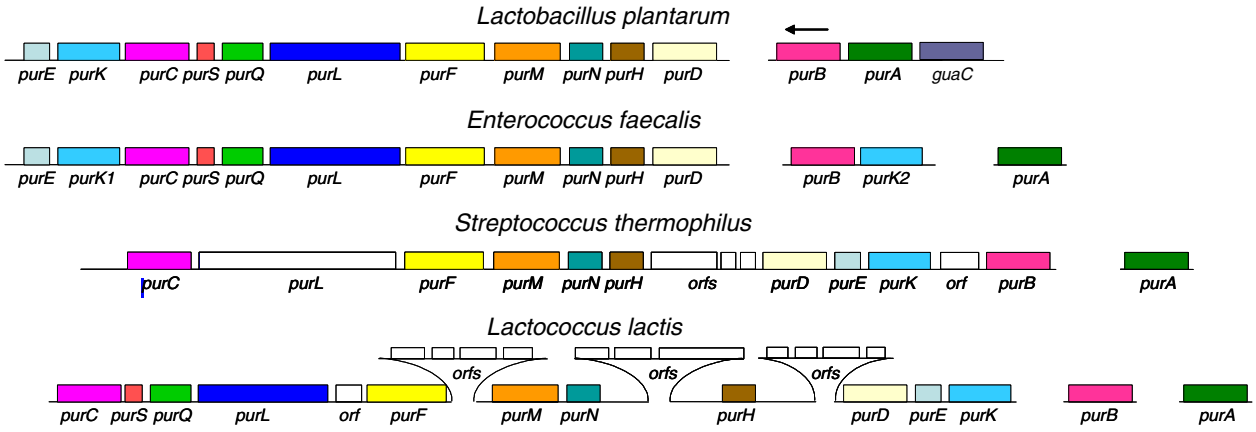

Fig. 10. Organization of the genes for the purine de novo synthesis in lactic acid bacteria. The following organisms are included: *Lb. plantarum* CCM1904, *E. faecalis* OG1RF, *S. thermophilus* CNRZ1066, and *L. lactis* subsp. *cremoris* MG1363. The genes encoding the following proteins are shown: *purA*, adenylosuccinate synthase; *purB*, adenylosuccinate lyase; *purC*, SAICAR synthase; *purD*, GAR synthase; *purEK*, CAIR synthase; *purF*, PRPP amidotransferase; *purH*, bifunctional AICAR transformylase/IMP cyclohydrolase; *purM*, AIR synthase; *purN*, GAR transformylase; *purQSL*, FGAM synthase. Please note that *purL* in *S. thermophilus* is larger and most probably encompass all functions required for FGAM synthase activity. Gene names followed by the numbers 1 and 2 indicate that two different alleles encoding proteins with similar function are present in the organism. All genes are transcribed from left to right, except when indicated otherwise by an arrow.

purMN, *purDEK* [81] and a separate *purH* gene, which may form an operon with the *hprT* gene of the purine salvage pathway [13]. *S. thermophilus* [82] appears to have an intermediary gene arrangement between the scattered *pur* clusters in *L. lactis* and the large operon structures of *Lb. plantarum* and *E. faecalis*. Quite distantly related, however, is the large *purL* gene, typical of gram-negative bacteria, instead of a shorter *purL* gene in combination with the *purS* and *purQ* genes [78]. Interestingly, both *Lb. plantarum* and *E. faecalis* possess *purK2* genes [37,79] encoding an additional ATPase subunit of the AIR carboxylase, but it is unknown whether both genes are functional. The carboxylation reaction does not require ATP hydrolysis per se, which is clear from the analysis of AIR carboxylases from higher organisms, but in lower branching organisms like bacteria, the extra energy consumption is maintained to speed up the reaction [71].

6.3. The PurR regulator and its DNA binding

PRPP is, as described above, a precursor of the IMP biosynthesis pathway. As a consequence the Pur enzymes are only required in the presence of sufficient supply of PRPP. In accordance with this rationale, PRPP has been found to act as a feed-forward inducer of transcription of the *pur* genes in the gram-positive model organism *B. subtilis*, and is believed to serve identical functions in all lactic acid bacteria (Fig. 11A). The sensor of PRPP availability in *B. subtilis* is the PurR repressor [83], which harbors a conserved PRPP binding domain. PurR binds to the promoter region of the *pur* operon in the absence of PRPP and prevents the RNA polymerase from binding to the promoter [84].

It was proposed that the PurR-mediated repression in *B. subtilis*, resulting from addition of purine bases to the growth medium, was due to a lowering of the cellular PRPP pool [84]. The explanation for the lowering followed two lines of evidence (Fig. 11A). First, the added bases exhausted the PRPP pool because of their efficient uptake and phosphoribosylation to the corresponding nucleotide monophosphate (Fig. 2). Second, because the PRPP synthase from *B. subtilis* is efficiently feedback inhibited by ADP [85], the accumulation of this substance after purine addition results in decreased PRPP production [84] (Fig. 11A).

In *L. lactis*, the homologue of the *B. subtilis* PurR repressor was identified as an activator of transcription [9]. The evidence came from the analysis of a *purR* mutant that required purines for growth, suggesting that PurR is a positive factor. This was confirmed by different kind of experiments. First, the purine requirement could be complemented *in trans* by introducing the *L. lactis purR* gene on a plasmid [9]. Second, introducing a point mutation in the PurR binding site (see below), resulted in reduced expression from a PurR-regulated promoter [86].

Comparative analysis showed that the PurR activator from *L. lactis* possessed PRPP and DNA binding domains very similar to the *B. subtilis* enzyme [9]. This strongly suggested that the PRPP pool also modulates the *L. lactis* PurR activator. Interestingly, a *purR*-encoded activator has so far only been detected in *L. lactis*. In the highly related *Streptococcus pneumoniae* a *purR* mutant was recently analyzed by use of DNA microarrays, and was by mutational analysis found to encode a transcriptional repressor [87]. In contrast to the parental strain, the *purR* mutant had constitutive high

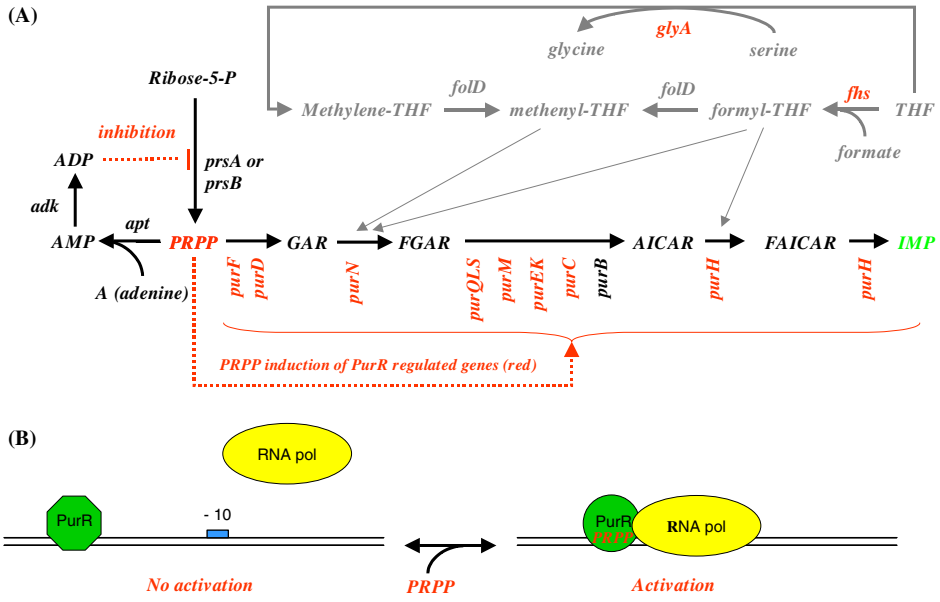

Fig. 11. Model for PurR-mediated regulation in *L. lactis*. (A) Control of the PRPP pool and genetic feed-forward regulation of the IMP biosynthetic pathway. According to the model for control of the PRPP level in *B. subtilis*, the PRPP pool can be diminished by phosphoribosylation of nucleobases to NMPs (here represented by adenine), and by ADP inhibition of the PRPP synthase. Under conditions of high PRPP levels PurR activates the transcription of the genes in the IMP biosynthesis pathway (shown in red), as well as the *glyA* and *fhs* genes involved in incorporation of C1 units from formate and serine into THF (tetrahydrofolate). The C1 units of formyl-THF and methanyl-THF are incorporated into purine bases by the PurN and PurH transformylases. (B) Model of PRPP induced transcription activation by PurR. PurR is bound to PurBox'es on the DNA under all conditions. Binding of PRPP changes the PurR conformation to recognize and position the RNA polymerase correctly with respect to the −10 region of the promoter, resulting in transcription initiation.

expression of all *pur* genes. PurR from *Lb. plantarum* most likely also functions as a repressor, since a *purR* mutation did not result in purine auxotrophy (F. Bringel and J. Martinussen, unpublished).

Fusion studies with the promoters for the *L. lactis purDEK* and *purCSQLF* operons inserted upstream of the promoterless *lacLM* operon, confirmed that purine addition resulted in lowering of the promoter activity also in *L. lactis* [81,86]. Because PurR in *L. lactis* is a transcriptional activator and not a repressor like the *B. subtilis* PurR, a model where PRPP binding prevents DNA binding was not plausible. Accordingly, purine addition had no effect on the transcription from the *purR* promoter, on which PurR acts as an autorepressor [9]. It was concluded that PurR from *L. lactis* binds to DNA independently of the PRPP concentration, and it was suggested that formation of a PRPP–PurR complex was required for interaction with the RNA polymerase, resulting in transcriptional activation at high PRPP concentrations (Fig. 11B) [9]. By comparing the *purDEK* and *purCSQLF* operon promoters, an invariant motif with the consensus sequence WWWCCGAACWWW, was detected exactly 75 bp upstream of the transcriptional start points in both promoters. Mutants in which the central G nucleotide of each motif was changed to a C residue were no longer PurR activated. In recognition of its importance for PurR activation, the motif was designated the PurBox [9]. PurBox'es have later been detected at conserved distances to the putative −10 promoter regions upstream of the *purMN*, and *purH* transcriptional units, as well as upstream of the *fsh* and *glyA* genes involved in supplying C1 units for the folate C1 pool (Fig. 11A) [4].

The definition of the *L. lactis* PurBox was crucial for the identification of the binding site for the *B. subtilis* PurR repressor. Studies had pointed at the importance of a GAAC-N24-GTTC motif, in the *B. subtilis purE-KBCSQLFMNHD* promoter region [88]. Binding of PurR to this region protected a very large region of DNA from DNAse I attack in a footprinting experiment, and introduced supercoiling, suggesting that the DNA is wrapped around PurR in the absence of PRPP [88]. The definition of the PurBox motif in *L. lactis* was reinforced by the identification of single PurBox'es also upstream of *B. subtilis pur* genes [9]. It was proposed that additional PurR repressors could be bound unspecifically in a head-to-tail direction to the DNA by strong contacts to the bound PurR repressor, forming the large protected regions. However a better explanation came from a bioinformatic analysis of the *B. subtilis* genome sequence, followed up by experimental verification [89]. This study reveled that two PurBox'es, directed head-to-head and spaced by 16–17 bp, were required for PRPP modulated PurR repression in *B. subtilis* [89]. In every case one of the PurBox'es was highly degenerated. Further analysis of PurR repressed

promoters in *B. subtilis* has recently shown that in order to be a functional PurR repressor site, one of the PurBox'es has to bind PurR weakly, while the other has to strongly bind PurR [90]. Two strong PurBox'es result in very efficient repression, but the strong DNA binding cannot be disrupted by PRPP [90].

A model for PRPP inhibited DNA binding, including one or two PurR dimers binding head-to-head to a DNA sequence with a strong and a weak PurBox, is readily comprehensible if the PRPP-induced conformational change required for disruption of the complex is relatively small. However, a much more complex and speculative model has been forwarded. It was postulated that a DNA bending region was present immediately outside the strong PurBox, and that PurR binding required that the DNA was bent at this position and wrapped around the strongly binding PurR monomer to obtain contact with an unspecific DNA binding region on its surface [90]. It was furthermore suggested that only this unspecific binding was modulated by PRPP. The model was prompted by the results from an analysis of the crystal structure of the *B. subtilis* His_6-tagged PurR dimer, which has been solved in the absence of DNA, in the presence [90] and absence [91] of PRPP bound to the PRPP binding pocket. Binding of PRPP induced only very subtle changes in the three-dimensional structure, with the major change being an arginine residue moving away from the PurR surface [90]. One might speculate whether the effect would be more accentuated if a PurR:DNA co-crystal with the wild-type PurR protein could be obtained and used for comparison.

Interestingly, not only the *B. subtilis*, but also the *L. lactis* PurR regulator may form complexes. An additional PurBox is present in the *purCSQLF* promoter, immediately upstream of the activating PurBox. These two tandem PurBox'es are required for high-level transcription of the operon but not for regulation, since full PurR-mediated regulation is retained at a 10% transcription level if the upstream PurBox is disrupted [86]. The *B. subtilis* and the *L. lactis* PurR proteins are thus able to form head-to-head and head-to-tail contacts, respectively.

6.4. The PurR regulon, analyzed by proteomics and DNA array analysis

In *L. lactis* the PurR regulon is induced by general purine starvation. When a *purD* mutant was allowed to exhaust its supply of the purine base hypoxanthine, its proteome showed increased synthesis of a number of proteins, when compared to the proteome of hypoxanthine-supplied cells [4]. The proteins were identified by mass spectrometric methods, and included the PurE, PurS, PurM, PurL, GlyA, and Fhs proteins. In a DNA microarray analysis of the transcriptomes of identical conditions using a partial array (M. Kilstrup and A. Nielsen, unpublished), increased mRNA levels from the *purC*, *purF*, *purH*, and *purM* genes was observed. The genes of all these proteins could be identified as members of the PurR regulon by the identification of PurBox motifs at the correct distance to putative promoter −10 regions in the *L. lactis* IL1403 genome sequence. Together with these, a number of additional potential PurR regulon members could be identified [4], including the *purA* gene and the *hprT*, *yriD* (*pbuO*), and *bmpA* genes. The PurR regulon, thus, appears to encompass at least the *purDEK*, *purCSQLF*, and *purMN* operons and the *glyA* and *fhs* genes as well as the putative *hprT-purH* operon. The *yriD* (*pbuO*) gene is also a likely member of the regulon as its homologue in *B. subtilis* encodes a guanine/hypoxanthine permease [89]. The function of the basic membrane protein encoded by *bmpA* has not been verified, but might be involved in nucleoside transport. In a DNA array analysis of *S. pneumoniae* the PurR regulon members were found to be induced by exposure to sublethal concentrations of the translation inhibitors erythromycin, chloramphenicol, and tetracycline, but not by streptomycin [87]. The mechanism or rationale for this regulation was not identified.

7. Conversion of IMP to AMP and GMP

AMP is synthesized from IMP by amination. The reaction is divided into two enzymatic steps catalyzed by PurA and PurB (Fig. 2). PurA catalyzes the condensation of the amino group of aspartate to IMP, forming succinyl-AMP (sAMP). Interestingly, GTP is hydrolyzed to GDP and inorganic phosphate in the process, functioning as a dehydrating agent [71]. PurB catalyzes the subsequent removal of fumarate from sAMP to form AMP. PurB is a bifunctional enzyme that catalyzes two reactions in the purine metabolism. In the synthesis of IMP, SAICAR is converted to AICAR by removal of fumarate, in a reaction also catalyzed by PurB. None of the enzymes have been purified or analyzed in gram-positive organisms. *E. coli* PurA is highly regulated through inhibition by AMP, GDP and ppGpp [71].

In the synthesis of GMP, IMP is first converted to XMP by oxidation with NAD^+ as the electron acceptor. The reaction is catalyzed by GuaB. XMP is subsequently aminated at the same position, using the amide group of glutamine as donor and ATP as energy donor. This reaction is catalyzed by GuaA. None of the enzymes have been purified or analyzed from gram-positive bacteria, but *E. coli* GuaB is inhibited by GMP and ppGpp [71]. *E. coli* GuaA is inhibited by the adenosine analogs psicofuranine and decoynine. GuaA from *L. lactis* is also inhibited by decoynine [4]. A separate enzyme, GuaC, catalyzes the deamination of GMP to IMP

thereby reversing the actions of GuaA and GuaB. To avoid a futile cycle, wasting both energy and amino groups, the two pathways are regulated independently.

7.1. Genetics of AMP and GMP formation

The *purA* and *purB* genes are transcribed individually in *L. lactis* [13]. The organization of the genes in *Lb. plantarum* [37] is interesting because the genes form a tight cluster with *guaC* required for IMP formation from GMP (Fig. 10). It seems that *purA* and *guaC* form an operon that is divergently transcribed relative to *purB* (Fig. 7). In *B. subtilis purB* is included in the *pur* operon [78], while the *purA* gene is transcribed alone. Yet again, *purB* of *E. faecalis* [79] is transcribed alone, while the *purA* gene appears to form an operon with *purK2* (Fig. 7) and possibly with genes involved in xanthine uptake and conversion to XMP.

While transcription of *purA* and *purB* are independently subjected to PurR repression in *B. subtilis*, only *purA* appears to be regulated in *L. lactis*. Interestingly, *purA* regulation in *L. lactis* appears to be opposite of that in *B. subtilis*, where purine addition results in *purA* repression [92]. In contrast, transcription of *purA* is decreased upon purine depletion of *L. lactis*. The transcription of *purB* is unaltered under these conditions (M. Kilstrup, unpublished). Also, *purA* expression is decreased during GTP starvation, through inhibition of GuaA with decoynine, while *purB* expression is unaltered (M. Kilstrup, unpublished). Nothing is known about the regulation of these genes in other lactic acid bacteria.

In *L. lactis*, *Lb. plantarum*, *E. faecalis*, and *B. subtilis* both *guaA* and *guaB* appear to be transcribed as separate genes. This is in contrast to the *guaC* gene, which appears to be clustered with different genes in the different organisms. In *L. lactis*, *guaC* is physically clustered with the *xpt* and *pbuX1* genes involved in xanthine uptake and conversion to XMP, possibly forming a *guaC–xpt–pbuX1* operon. As mentioned in relation to the AMP synthesis pathway, *guaC* is clustered with *purA* and *purB* in *Lb. plantarum*, most likely forming a *purA–guaC* operon, divergently transcribed relative to *purB*. In *E. faecalis*, the *guaC* gene appears to be the last gene in an operon with the genes for a xanthine permease and a putative guanine or cytosine deaminase. *B. subtilis guaC* is not clustered together with any gene involved in purine metabolism.

Purine depletion of *L. lactis purD* decreases transcription of both *guaA* and *guaB* to around 20% of the normal level, while *guaC* and all de novo genes are over-expressed (M. Kilstrup, unpublished). The *guaC* gene is, however, only marginally up-regulated. The rationale for the down-regulation of *guaA* and *guaB* is not obvious, but since *purA* expression was also down-regulated under these conditions, the regulation could involve some kind of feed forward control, ensuring that the two branches using IMP are only active when their common substrate is present. Upon decoynine addition, which results in GTP starvation and possibly IMP accumulation, *guaA* and *guaB* expression is increased while *guaC* expression is decreased like all de novo genes (M. Kilstrup, unpublished). The *guaC* gene acts very much like a PurR-regulated gene, and the *guaC* promoter region contains a degenerate PurBox (TTTCCGAATGATA) located at the conserved distance to the putative −10 box (TAAAAT). It is not known whether this structure is responsible for purine regulation. *B. subtilis guaC* expression is under PurR repression control [89], while *guaA* and guaB are virtually unregulated [92]. Marginal repression was seen for *B. subtilis guaB* in the presence of exogenous guanosine, but not by adenine [92].

8. Purine nucleotide interconversions

8.1. NDP synthesis

ADP and GDP are formed from their corresponding nucleotide monophosphates by the action of specific kinases. The Adk (adenylate kinase) enzyme produces two ADP molecules from AMP and ATP, whereas Gmk (guanylate kinase) produces GDP and ADP from GMP and ATP. The Adk enzyme has been crystallized from three *Bacillus* species [93], but the kinetic properties of bacterial adenylate kinases have only been studied in detail for the *E. coli* enzyme [94–96]. The *E. coli* enzyme is reversible and has a broad specificity for its NTP substrate and will use GTP, CTP or UTP as donor at 7–20% of the rate with ATP [94]. Its specificity for AMP, however, appears to be absolute [94]. The reversibility and its broad specificity have interesting implications, which will be discussed below. *E. coli* Gmk is specific for its phosphate donor ATP [97].

Both *adk* and *gmk* appear to be transcribed separately in *L. lactis*, *Lb. plantarum*, *E. faecalis*, and *B. subtilis*. The *L. lactis* genes appear to be regulated in a coordinated way. Like *purA*, *guaA*, and *guaB*, they are severely down-regulated upon general purine depletion but in contrast to the genes in the branching pathways, the *adk* and *gmk* genes are unaffected by decoynine addition (M. Kilstrup, unpublished).

8.2. NTP synthesis

ATP is formed at two points in glycolysis, through the action of phosphoglycerate kinase (Pgk) and pyruvate kinase (Pyk). These reactions are likely to be the main sources of ATP under normal conditions. The lack of respiration under normal growth conditions makes

the free-energy metabolism of lactic acid bacteria quite simple compared to aerobic bacteria because virtually all ATP regeneration results from substrate level phosphorylation. The stoichiometry at which ATP is produced from sugar varies to some extent depending on the growth conditions: When sugar is present in excess in the growth medium sugar degradation takes place through the homo- or hetero-fermentative pathway [98]; under sugar limiting conditions, the metabolism of pyruvate may shift to production of a mixture of acids including acetate and the yield of ATP may theoretically increase to three moles of ADP converted to ATP per mole of glucose.

In almost all organisms analyzed so far, the formation of NTP from NDP is catalyzed by an NDP kinase (Ndk) [99] that uses any NTP as the phosphate donor, and any NDP as phosphate acceptor, thus equilibrating the NDP/NTP ratios in the cell. In contrast, *L. lactis* [13] and *E. faecalis* [79], do not possess a nucleotide diphosphate kinase (Ndk). It was previously believed that the Ndk enzyme was indispensable, because of its central role in the nucleotide metabolism, but studies of *ndk* mutants have shown that other enzymes have overlapping activities [99,100]. In an *ndk* mutant of *E. coli* it was found that the adenylate kinase could compensate for the mutation [101]. The purified Adk enzyme was, aside from its AMP kinase activity, capable of converting any NDP to its corresponding NTP by use of ATP as phosphate donor. Also, the glycolytic enzyme pyruvate kinase (Pyk) has been shown to be a candidate for a PEP-dependent NDP kinase activity in *E. coli* [102]. Pyk is a likely NDP kinase candidate also in *L. lactis*, because lactococcal Pyk has a broad substrate specificity and phosphorylates both ADP and GDP efficiently, with GDP as the preferred substrate [103]. We have evidence that Pyk is responsible for a substantial fraction of the GDP kinase activity in *L. lactis* (M. Kilstrup unpublished). A third enzyme has been reported to possess NDP kinase activity. The polyphosphate kinases of *E. coli* and *Pseudomonas aeruginosa* may use any NDP as substrate in the reverse reaction and produce NTP at the expense of the terminal phosphate of a polyphosphate [104]. In *L. lactis* this possibility is not an option, since the genome of this organism does not encode a polyphosphate kinase [13].

While Ndk is absent in *L. lactis* and *E. faecalis*, it is present in other lactic acid bacteria. In spite of its apparent promiscuous substrate selectivity, the specificity of Ndk is controlled in the organisms where it has been under investigation, i.e., in *E. coli*, *P. aeruginosa*, and *Mycobacterium tuberculosis* [99]. It has been found that under conditions of GTP deprivation in *P. aeruginosa*, the GTP sensing protein Pra/Era of the GTPase switch protein family (Fig. 12), combines with Ndk and changes its specificity to preferentially function as a GDP kinase [99,105]. GTP is used in large amounts in the bacterium for generation of GDP–mannose for alginate synthesis.

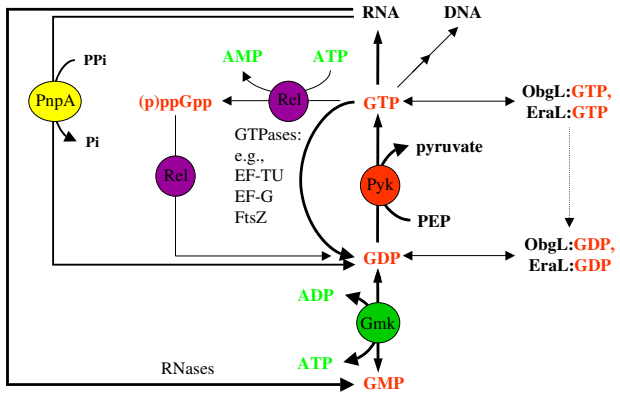

Fig. 12. Guanine nucleotide interconversions in *L. lactis*. The different pathways for guanine nucleotide interconversions are shown. The enzymes are shown by their genetically derived names as follows: Pyk, pyruvate kinase; Gmk, guanylate kinase; Rel, (p)ppGpp synthase (ATP,GTP 3′ pyrophosphotransferase); PnpA, polyribonucleotide nucleotidyltransferase; ObgL, GTP-binding protein of the GTP1/OBG family; and EraL, GTP-binding protein of the Era/ThdF family. The expected importance of the pathways for the cellular GTP, GDP, and GMP concentrations is shown by the thickness of the arrows.

9. NTP utilization

The fluxes from ATP and GTP are highly branched and the mechanisms for using these high-energy compounds are diverse, as the nucleotides function both as energy donors, phosphate donors, and substrates for polymerization (see GTP in Fig. 12). When the nucleotides function as energy and phosphate donors, the nucleotide moiety is returned to the nucleotide pool at the mono or diphosphate level to be re-utilized. Also, when NTPs are used as substrates for RNA synthesis, they will eventually return as NMPs after normal RNA degradation. So mRNA, and in rare cases rRNA [106], may be considered as a reservoir of nucleotides. DNA may be considered a stable reservoir for deoxyribonucleotides except during conditions of DNA damage and repair. Furthermore the deoxynucleotide dTTP is utilized for synthesis of cofactors in exopolysaccharide synthesis, e.g., dTDP–rhamnose [107].

9.1. Consumption of ATP

ATP is the general energy currency in lactic acid bacteria and numerous ATPases are present in the cells. One of most expensive ATPase reactions in the cell is the translocation of protons across the cytoplasmic membrane catalyzed by the H^+-ATPase. This process aids in maintaining the intracellular pH and generates a proton gradient across the cytoplasmic membrane, which can be used as the driving force for numerous

active transport processes in and out of the cells. Because lactic acid bacteria are usually unable to respire, the H^+-ATPase is an essential enzyme for growth under normal growth conditions [108]. However, in the presence of a hemin source, *L. lactis* produces active cytochromes and engages in active respiration [109,110]. Under these conditions the H^+-ATPase is no longer required for growth and the reconstituted respiratory processes leads to translocation of protons out of the cell [111]. Interestingly, when cells are provided with conditions for respiration and are simultaneously starved for sugar (by reducing the sugar uptake), the ratio between the intracellular pool of ATP and ADP becomes sufficiently low to allow for reversal of the H^+-ATPase reaction, resulting in oxidative phosphorylation [112]. The yield of ATP per mole of consumed hexose sugar can increase significantly, and the biomass yield on glucose is more than doubled compared to homolactic fermentation.

The intracellular pools of ATP and ADP also play an important role in the regulation of glycolysis: the metabolic flux through glycolysis in *E. coli* is very sensitive to changes in the intracellular pools of ATP and ADP: when the ATP/ADP ratio dropped upon introduction of an additional ATP consuming reaction glycolysis increased, which shows that glycolysis is controlled almost exclusively by the demand for ATP [113,114]. In *L. lactis* the response towards lowering of the ATP/ADP ratio depended on the actual conditions for growth: The flux increased in slow or non-growing cells, but it was unchanged in rapidly growing cells [115,116].

9.2. Consumption of GTP

While ATP appears to be the general fluctuating household energy currency, GTP is used in the more basic energy requiring reactions where a steady supply of energy is required. It has been calculated that protein synthesis in lactococcal cells requires around 65% of all synthesized ATP equivalents, when it is supplemented with amino acids and vitamins [117]. Half of the energy is required for activation of amino acids into aa-tRNA (ATP → AMP) and the other half for polymerization into proteins. Interestingly, the energy consumption of the protein synthesis machinery is in the form of GTP, which is utilized by the elongation factors EF-Tu and EF-G. The GTP hydrolysis by the elongation factors is a slow process, which permits testing of several aminoacyl-tRNA before incorporation of the amino acid into the polypeptide chain [118]. GTP hydrolysis for protein synthesis alone, thus, accounts for more than 32% of all the energy a lactic acid bacterium uses in its metabolism.

Under amino acid starvation, GTP is converted to the regulatory derivative pppGpp by pyrophosphate transfer from ATP [119,120]. In *E. coli* this reaction is catalyzed by the ribosomally associated protein RelA, which produces (p)ppGpp in the presence of bound un-acylated transfer RNA. In this organism, another (p)ppGpp forming enzyme, SpoT, catalyses the same reaction in response to the growth rate [121]. SpoT was also shown to possess a ppGpp degrading activity [122]. Gram-positive bacteria possess a single protein, Rel, which exhibit features of both RelA and SpoT proteins [123–125].

9.3. Consumption of pyrimidine nucleotides

Besides their roles as precursors for RNA and DNA, pyrimidine nucleotides have important roles in the biosynthesis of components present in the cell envelope, including peptidoglycan and exopolysaccharides (EPS). *N*-Acetyl-glucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) constitute the back-bone of peptidoglycan in lactic acid bacteria. The sugars are activated by UTP yielding UDP–GlcNAc that is subsequently reduced to UDP–MurNAc (for a review, see [126]). Lipoteichoic acid rather than teichoic acid is abundantly found in lactic acid bacteria (reviewed in [127]). The glycerol derivative constituting the backbone of lipoteichoic acid is activated by CTP to obtain CDP–diacylglycerol, which is also an intermediate in the synthesis of phospholipids.

Considering their contribution to the texture of fermented dairy products, EPS production by lactic acid bacteria is of special interest. Again, hexoses used for EPS polymerization are activated by UDP linkage. Central metabolites are UDP–glucose and UDP–galactose. Rhamnose is also a major component of EPS, and the precursor in the polymerization is dTDP–rhamnose (for a review, see [128]). It should be emphasized that the pyrimidine nucleotides utilized in sugar metabolism are fed back into the nucleotide interconversion pathway again, so there is no drain from the pyrimidine biosynthesis. The nucleotides are recycled both as mono- and dinucleotides, depending on whether the sugar is incorporated with or without phosphate during the polymerization process. Since different nucleotides are used in the activation of sugars, changing nucleotide concentrations may potentially influence both the rate of production and the composition of polysaccharides.

10. Salvage pathways

External nucleotides arise in nature by degradation of nucleic acids. The nucleotides cannot be incorporated as such but must first be dephosphorylated externally by nucleotidases to the corresponding nucleoside (Fig. 13, reaction 9) as found in *L. lactis* (J. Martinussen, unpublished). The nucleosides are then transported into the cell (reaction 5) followed by either phosphorylation into

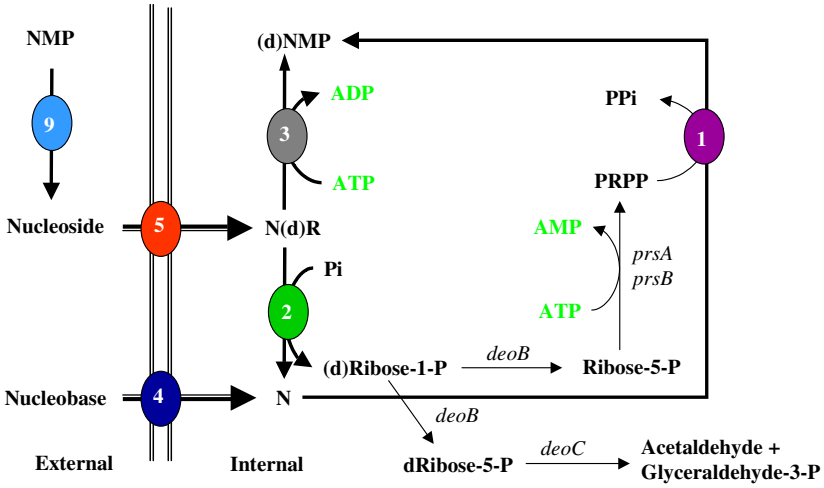

Fig. 13. The salvage pathways of external nucleotides, nucleosides, and nucleobases in organisms with nucleoside phosphorylases, e.g., in *L. lactis*. Nucleobases and nucleosides are taken up directly by dedicated transporters (4 and 5, respectively). External nucleotides are dephosphorylated by a phosphatase (9) and subsequently transported into the cell (5). Nucleobases are phosphoribosylated to the corresponding NMP by phosphoribosyltransferases (1). Nucleosides are either phosphorolytically cleaved to the nucleobase and a ribose or deoxyribose moiety by the purine or pyrimidine phosphorylase (2), or phosphorylated directly to the NMP or dNMP by the corresponding kinase (3). Ribose-1-phosphate obtained by action of the phosphorylases is converted to ribose-5-phosphate by (DeoB) and salvaged into PRPP (PrsA or PrsB) and used in nucleotide formation. The deoxyribose-1-phosphate (dRibose-1-P) formed from the phosphorolysis of the deoxynucleosides enters glycolysis after conversion to dRibose-5-P by DeoB to acetaldehyde and glyceraldehydes-3-phosphate by DeoC. Enzymes encoded by the genes in alphabetical order: *deoB*, phosphopentomutase; *deoC*, deoxyriboaldolase; *prs*, PRPP synthase.

the corresponding nucleotide by a kinase (reaction 3), or degradation to the nucleobase and pentose sugar by a nucleoside phosphorylase (reaction 2). In certain species ribonucleosides are degraded by hydrolases (Fig. 14, reaction 7) instead of Phosphorylases. Finally, the base is converted to the NMP by a phosphoribosyltransferase (reaction 1 in Figs. 13 and 14). Nucleobases present in the medium are also transported into the cell (reaction 4) followed by conversion to NMP by the phosphoribosyltransferases. Transporters for nucleobases have been identified in a few cases. Some bacteria such as *E. coli* utilize nucleosides very efficiently as carbon and energy sources by degrading the pentose moiety of the nucleoside. Also the amino group in cytidine and adenosine may be utilized as nitrogen source without degradation of the purine or pyrimidine rings (for review see [129]). Many lactic acid bacteria have both cytidine and adenosine deaminase but since they usually require many amino acids the contribution of deamination of these nucleosides has been difficult to estimate. Nucleosides have been tested as sole energy source in lactococci but no growth was observed in the absence of glucose (K. Hammer and J. Martinussen, unpublished). Therefore, the main function of the salvage pathways in lactic acid bacteria seems to be to rescue nucleobases or nucleosides for nucleotide synthesis. In addition, the rescue of deoxynucleosides for DNA synthesis is vital for some lactobacilli. In the following the salvage pathways for *L. lactis* (Fig. 13) is taken as a model for the salvage pathway systems containing nucleoside phosphorylases, which is the common mode of salvage. An overview of the specificities of the identified transporters and enzymes for each nucleoside and nucleobase are shown in Tables 1 and 2. This is then followed by a description of the salvage pathways present in species of

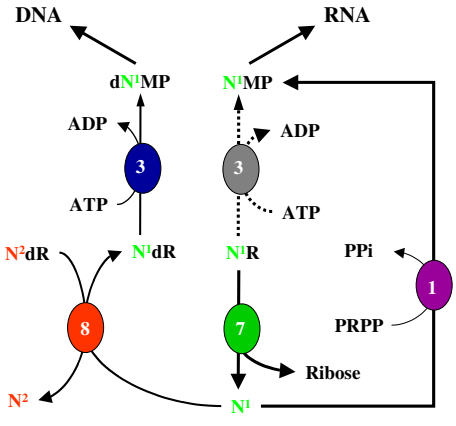

Fig. 14. *trans-N*-Deoxyribosylase-dependent nucleotide synthesis in lactobacilli. The key reaction in organisms using *trans-N*-deoxyribosylases (8) is the exchange of a nucleobase between a deoxyribonucleoside (N^2dR) and a free nucleobase N^1, resulting in the formation of a new product N^1dR. The product is then phosphorylated (3) to the corresponding dN^1TP. The free nucleobase N^1 may be obtained by hydrolysis of the corresponding nucleoside N^1R. This reaction (7) is catalyzed by a ribonucleoside hydrolase. The ribose moiety can be used in carbon metabolism. The base N^2 produced during the action of *trans-N*-deoxyribosylase may be salvaged by a phosphoribosyl transferase (1) yielding the corresponding NMP. Also, ribonucleoside kinases may be present that phosphorylate ribonucleosides into NMP (also named 3; note that these enzymes are different from the deoxyribonucleoside kinases).

Table 1
Substrate specificity of nucleoside transport and salvage reactions in *L. lactis*

Nucleoside	Transport			Phosphorylases		Kinases		
	I	II	III	Pup	Pdp	Udk	Tdk	DukAB
GR	+	–		+	–			
AR	+	–		+	–			
IR	+	–		+	–			
XR	(+)			–	–			
UR	–	+		–	+	+	–	
CR	+	–				+	–	
GdR	+	–		+	–			(+)
AdR	+	–		+	–			(+)
IdR	+	–		+	–			(+)
UdR	–	+		–	+	–	+	
TdR	–	–	+	–	+		+	
CdR	+	–						(+)

Transport system I is encoded by *nupABC*, the genes encoding transport system II and III have not been identified. Pup, purine nucleoside phosphorylase; Pdp, pyrimidine nucleoside phosphorylase; Udk, uridine/cytidine kinase; DukAB, deoxynucleoside kinase; Tdk, thymidine kinase GR, guanosine, AR, adenosine, IR, inosine, XR, xanthosine, UR, uridine, CR, cytidine, GdR, deoxyguanosine, AdR, deoxyadenosine, IdR, deoxyinosine, UdR, deoxyuridine, TdR, thymidine, CdR, deoxycytidine. + and – represents the presence and absence of the activity, respectively. Brackets indicate presumed substrate specificity, not based on experimental data.

Table 2
Substrate specificity of nucleobase transport and salvage reactions in *L. lactis*

Base	Transport		Phosphoribosyl transferases					
	PyrP	PbuX	Hpt	Apt	Xpt	Upp	PyrE	HprT
G			+					(+)
A				(+)				
Hx			+					(+)
X		(+)			(+)			
U	+					+	–	
C								
O						–	+	
T						–		

PurP, uracil permease; PbuX, xanthine permease; Hpt, hypoxanthine–guanine phosphoribosyltransferase; Apt, adenine phosphoribosyltransferase; Xpt, xanthine phosphoribosyltransferase; Upp, uracil phosphoribosyltransferase; Pyr E, orotate phosphoribosyltransferase; HprT, annotated as hypoxanthine–guanine phosphoribosyltransferase; G, guanine; A, adenine; Hx, hypoxanthine; X, xanthine; U, uracil; C, cytosine; O, orotate; T, thymine. + and – represents the presence and absence of the activity, respectively. Brackets indicate presumed substrate specificity, not based on experimental data.

Lactobacilli lacking nucleoside phosphorylases but containing *trans-N*-deoxyribosyltransferases (Fig. 14). This kind of salvage system is usually found in species auxotrophic for a deoxyribonucleoside due to a non-functional ribonucleotide reductase.

10.1. Nucleobase transport

A number of transport systems are involved in the uptake of the nucleobases (reaction 4 in Fig. 13). For uracil a high affinity uptake system encoded by *pyrP* has been identified by the phenotype of the *pyrP* mutant in *L. lactis* MG1363 [15]. A limited number of *L. lactis* strains have been shown to encode a functional orotate transporter *oroP* (E. Defoor and J. Martinussen, unpublished). Orotate is the fourth intermediate in pyrimidine biosynthesis (Fig. 3). This observation is of particular interest, since milk contains orotate in significant amounts. The uptake system is discussed in Section 13.1. From the genome sequence of *L. lactis* IL1403 the gene products of the *pbuX* and *yriD*/*pbuO* genes are expected to be involved in the transport of xanthine and guanine + hypoxanthine, respectively as the homologues from *B. subtilis* [130]. Other transport systems are expected to exist, e.g., an adenine transporter, but have not yet been identified.

10.2. Phosphoribosyltransferases

The salvage of the nucleobases is performed by conversion to ribonucleoside monophosphates through the action of phosphoribosyltransferases (reaction 1 in Fig. 13). As shown in Figs. 2 and 3, all common bases, except cytosine and thymine, are salvaged through this

type of reaction using enzymes with different substrate specificity (Table 2). Uracil phosphoribosyltransferase (Upp) encoded by the *upp* gene has only uracil as substrate. This is in accordance with the property of the homologous enzymes from other organisms. Mutants in *upp* have been isolated in *L. lactis* and it was shown that a *pyrF upp* double mutant could not grow with uracil as pyrimidine source, while uridine supported growth [131]. A general purine requiring mutant of *L. lactis* CHCC373 was able to grow with any of the four purine bases, adenine, guanine, hypoxanthine and xanthine [132]. Therefore *L. lactis* most likely possess phosphoribosyltransferase activities acting on all four purine bases, and three of these have been demonstrated by enzyme assays [132] (xanthine was not tested). In the *L. lactis* IL1403 genome four genes have been annotated as purine phosphoribosyltransferase genes: *hpt*, *xpt*, *apt*, and *hprT*. Cloning and complementation of an *E. coli* mutant has shown that the *hpt* gene encodes a hypoxanthine guanine phosphoribosyltransferase [132]. *B. subtilis xpt* encodes a xanthine specific phosphoribosyltransferase [130]. The *L. lactis xpt* gene is located downstream of *guaC* and immediately upstream of *pbuX* indicating that the gene product is involved in xanthine metabolism. The adenine phosphoribosyltransferase is most likely encoded by the *apt* gene while the *hprT* gene is annotated as a gene for a secondary hypoxanthine and guanine phosphoribosyltransferase, resembling *hpt*.

10.3. Nucleoside transport

Auxotrophic *pur* or *pyr* mutants can grow if the proper nucleosides are present in the growth medium. In order to utilize these, the nucleosides have to be transported across the cytoplasmic membrane (reaction 5 in Fig. 13). At least two different nucleoside uptake systems have been identified in *L. lactis*. One system is responsible for the uptake of cytidine, deoxycytidine and all common purine nucleosides, whereas another is responsible for the uptake of uridine and deoxyuridine [8]. The uptake system for purines and cytidine has been identified by IS*S1* mutagenesis and nucleotide sequence determination of the mutant locus. The uptake system is of the ABC transporter type, and the genes were originally annotated as *yngEFG* in the *L. lactis* chromosome, but are now named *nupABC* (J. Martinussen, unpublished). *L. lactis* also assimilates thymidine [115], and since neither uridine nor inosine uptake was inhibited by thymidine, a third nucleoside transport system responsible for thymidine uptake must be present (Table 1) [8] (J. Martinussen, unpublished).

10.4. Nucleoside deaminases

In order to utilize adenosine and cytidine as general purine and pyrimidine sources, the two compounds have to be deaminated to obtain inosine and uridine, respectively (Figs. 2 and 3). Both *cdd*, encoding cytidine deaminase, and *add* encoding adenosine deaminase are present in the *L. lactis* IL1403 genome. The presence of a functional cytidine deaminase with activity towards both deoxycytidine and cytidine has been verified by mutational studies [133].

10.5. Nucleoside phosphorylases

Nucleoside phosphorylases catalyze the phosphorolytic cleavage of the nucleoside resulting in the formation of the free nucleobase and ribose-1-phosphate (Ribose-1-P) or deoxyribose-1-phosphate (dRibose-1-P) (reaction 2 in Fig. 13). These enzymes are found in most organisms from bacteria to man. According to their substrate specificity they are divided into purine nucleoside phosphorylases (Pup) and pyrimidine nucleoside phosphorylases (Pdp). The Pup enzymes have both ribo- and deoxyribonucleosides as substrates, while this is not usually the case for Pdp enzymes. Many organisms, including *E. coli*, therefore contain both a thymidine phosphorylase (DeoA), specific for thymidine and deoxyuridine, and a uridine phosphorylase with uridine as the preferred substrate (Udp). The cytosine containing nucleosides, however, are not substrates for any of the known pyrimidine nucleoside phosphorylases. In *L. lactis* MG1363 a single Pdp enzyme, with activity towards U, UR, UdR, T, and TdR was identified by mutational studies (Table 1) [133].

Pup enzyme activities have been identified in *L. lactis* MG1363 towards inosine, adenosine, guanosine and the corresponding deoxyribonucleosides. All activities were lost in the *pup* mutant [133] (K. Hammer unpublished) and therefore, in contrast to *B. subtilis*, only one Pup enzyme is present in *L. lactis*. *B. subtilis* contains both an inosine/guanosine phosphorylase and an adenosine phosphorylase [134]. In *E. coli* a second Pup enzyme (XapA) is only present in cells growing with xanthosine [135]. The nucleotide sequence of the mutated *pup* locus has not been determined, but it most likely corresponds to *deoD* of *L. lactis* IL1403. Historically there is some controversy in the naming of the genes in *E. coli* and *B. subtilis*. In *E. coli* the gene was first named *pup* and then changed to *deoD* as it was found as the fourth gene in the *deoCABD* operon, involved in deoxyribose metabolism [136]. For a review see [137]. In *B. subtilis*, however the gene has been designated *pup*, a more appropriate designation. The *pup* (*deoD*) gene is found in a *deoB–yjfG–pup* (*deoD*) cluster. The phenotype of an insertional mutation in *deoB* suggested that the *pup* gene was no longer expressed, indicating that the genes form an operon [3,138].

A *udp* gene is annotated in the *L. lactis* IL1403 genome sequence [13]. Since mutational studies have shown that *L. lactis* MG1363 does not possess a uridine specific

phosphorylase, (i) the annotation could be wrong, (ii) the gene is inactive, or (iii) IL1403 is different from MG1363 in this respect. Since the transcription of the gene is induced during purine starvation (M. Kilstrup, unpublished) and the amino acid sequence also shows similarity to a large family of Purine Phosphorylases, it is possible that the gene encodes a purine specific phosphorylase, e.g., a xanthosine phosphorylase not yet identified in *L. lactis*.

10.6. Nucleoside synthesis

The Pup- and Pdp-catalyzed reactions are thermodynamically reversible, but the low concentration of Ribose-1-P makes the ribonucleoside synthesis reactions so unfavorable under normal physiological conditions that they appear irreversible. In Fig. 3, the arrow symbolizing the Pdp catalyzed conversion between UR and U is therefore only shown in the direction towards U. The irreversibility of Pdp in *L. lactis* was shown by the inability of U to satisfy the pyrimidine requirement of a *upp pyrF* mutant [131]. However, thymine, which cannot be utilized by wild-type bacteria of many different species, can be converted to thymidine by Pdp (DeoA) in thymine requiring mutants, where the dRibose-1-P pools are high [139]. When supplied with purine ribonucleosides as a source of Ribose-1-P, the uracil analogue 5-fluorouracil (FU) is converted to 5-fluorouridine (FUR) by Pdp in *L. lactis*. This was shown by the FU sensitivity of *upp*, *tdk* mutants in the presence of adenosine, but not in its absence [133].

L. lactis appears to contain sufficient levels of dRibose-1-P for reversing the Pdp reaction towards NdR synthesis because both *pdp* and *tdk* mutations could be selected in a *upp* mutant background in *L. lactis* MG1363, using high concentrations of FU. High concentrations of FU were required because *upp* mutants are resistant to low FU concentrations. The increased resistance by the *pdp* mutation indicated that FU could be converted to either FUR or 5-fluoro-deoxyuridine (FUdR) in a Pdp^+ strain. The resistance from the *tdk* mutation showed that FUdR was synthesized and should be converted to 5-fluoro-deoxy-UMP (FdUMP) by the Tdk enzyme for toxicity (see also the section on mutant phenotypes). Thymine was found to be unable to serve as a source for dTTP in the two *thyA* mutants so far isolated [115,140]. This observation is discussed under mutant phenotypes.

The Pup enzyme appears to be able to convert adenine to adenosine using Ribose-1-P at the low internal concentration as adenine can be used as a general purine source in a *purD* mutant, but not in a *purD*, *pup* double mutant (K. Hammer unpublished). A part of the adenine has to be converted to IMP in order to serve as precursor for GMP synthesis (Fig. 2). The Pup-dependent reaction pathway in the *purD* mutant is therefore suggested to be: A → AR → IR → Hx → IMP.

10.7. Nucleoside kinases

One kinase for ribonucleosides has been identified in *L. lactis*. Uridine kinase (Udk) can convert both UR and CR to UMP and CMP, respectively, as shown by mutational studies [133]. After uptake of uridine it may either be degraded by Pdp to uracil and Ribose-1-P or converted to UMP by Udk. Cytidine is not a substrate for any phosphorylase, but it is either converted to CMP by Udk or deaminated to uridine by cytidine deaminase (Cdd) (Fig. 3).

The absence of purine ribonucleoside kinases in *L. lactis* has been supported by growth experiments using a *purD pup* double mutant (Karin Hammer, unpublished). Only the purine bases could support growth in this mutant in contrast to the parent *purD* strain, which could also utilize the purine ribonucleosides. Thus the only pathway for purine ribonucleoside salvage in Lactococcus is through phosphorolytic cleavage by Pup to the free nucleobase and Ribose-1-P (Fig. 2).

Deoxyribonucleoside kinases are found in *L. lactis* (Figs. 2 and 3) indicating that the natural deoxyribonucleosides may be converted into the corresponding mononucleotides in *L. lactis* as found in *B. subtilis*. Thymidine kinase (Tdk) has both thymidine and deoxyuridine as substrates, as verified by mutational analysis [133]. The genome of *L. lactis* IL1403 specifies the presence of DukA and DukB, which are members of the family of deoxyadenosine/deoxyguanosine kinases. Deoxycytidine kinase is encoded by the gene for deoxyguanosine kinase in lactobacilli [141]. This may also be the case in lactococci.

10.8. Salvage gene localization and regulation of gene expression

Despite the fact that most of the salvage genes are scattered around the bacterial chromosome, some organizational patterns seem to be preserved in lactic acid bacteria and related bacteria. In particular this applies for the genes of the three different phosphoribosyltransferases as well as those of some of the enzymes degrading the nucleosides (Table 3). A physical clustering of *xpt* (X → XMP) and *pbuX* encoding the permease for xanthine, is very common in lactic acid bacteria. In some cases, *guaC* required for the conversion from GMP to IMP (Fig. 2), is included in the genetic cluster. The preservation of a genetic clustering of *ftsH*, encoding a chaperone/ATPase-dependent protease involved in the cell division, with *hpt* (Hx → IMP) is very interesting as well as the frequent location of *recJ*, encoding a ssDNA specific exonuclease next to *apt*

Table 3
Conserved gene order of salvage genes in related gram-positive bacteria: *B. subtilis* strain 168, *E. faecalis* V583, *L. lactis* IL1403, *Lb. plantarum* WCFS1, *S. pyogenes* M1 GAS, and *S. thermophilus* LMG18311

Genes	Encoded proteins	Other neighbour genes or comments	Bacteria
xpt, *pbuX*	Xanthine phosphoribosyltransferase (*xpt*); xanthine permease (*pbuX*)	*guaC* in bacteria[a] *purB*[b] *purK*[b] *xpt* disrupted in *S. thermophilus*	*L. lactis*[a]; *S. thermophilus*; *S. pyogenes*[a]; *B. subtilis*; *E. faecalis*[b]; *E. faecalis* has a second *pbuX* gene in a cluster with *guaC*. In *Lb. plantarum* a membrane protein encoding gene is neighbour to *xpt*
hpt, *ftsH*	Hypoxanthine/guanine phosphoribosyltransferase (*hpt*); ATP dependent Zn protease (*ftsH*) *hpt* is also named *hprT* in some bacteria. *L.lactis* has both an *hpt* and an *hprT* gene	*hsp33* in bacteria[c] *ftsH* is also called *hflB* in *E. coli*	*L. lactis*; *S. thermophilus*; *Lb. Plantarum*[c]; *B. subtilis*[c]; *E. faecalis*[c]
recJ, *apt*	ssDNA specific exonuclease (*recJ*); adenine phosphoribosyl-transferase (*apt*)		*L. lactis*; *S. thermophilus*; *Lb. plantarum*; *S. pyogenes*; *E. faecalis*
pyrP in *pyr* operon	Uracil permease (*pyrP*); proteins involved in Pyr-biosynthesis		*L. lactis*; *S. thermophilus*; *B. subtilis*; *S. pyogenes*; *E. faecalis*
cdd, *deoC*, *pdp*	Cytidine deaminase (*cdd*); deoxyriboaldolase (*deoC*), pyrimidine nucleoside phosphorylase (*pdp*)	*bmpA*, possible nucleoside transporter in *L. lactis*. *DeoC* and *pdp* are disrupted in *S. thermophilus*	*L. lactis*; *S. thermophilus*; *E. faecalis*. See also next line below
deoC, *nupC*, *pdp*	Deoxyriboaldolase (*deoC*), nucleoside transporter (*nupC*); pyrimidine nucleoside phosphorylase (*pdp*)	*deoR* upstream in *B. subtilis*. Other regulator in *S. pyogenes*	*B. subtilis*; Gene order opposite in *S. pyogenes* in which *pdp* is named *udp*
deoB, *orf*, *pup*	Phosphopentomutase (*deoB* also *drm*); sometimes an ORF; purine nucleoside phosphorylase (*pup* also *deoD* or *punA*)	Both *deoD* and *punA* in bacteria[d]. *rpiA* in bacteria[e]	*L. lactis*; *S. thermophilus*[de]; *S. pyogenes*[de]; *B. subtilis*; *E. faecalis*[d]

Bacteria labeled a, b, c, d or e have the correspondingly marked gene in the gene cluster.
Gene symbols not explained in the Table: *hsp33*: Heat shock protein 33 (disulfide bond chaperone); *rpiA*: ribose-phosphate isomerase. *deoR*: regulatory gene for *deoC* operon.

(A → AMP). These clusters, especially if the genes form operons, suggests a relation between nucleobase salvage, cell division (*ftsH*) and DNA recombination/repair (*recJ*). The need for additional purine bases during DNA repair is also indicated by the phenotype of insertional *deoB* mutants during DNA damage induced by presence of mitomycin C [138].

The enzymes responsible for degradation of almost all nucleosides in *E. coli* are specified by the *deo*-operon [137]. Instead of this large genetic unit, two separate gene clusters are present in many of the low GC gram-positive bacteria including lactic acid bacteria. One cluster consists of *pdp* (*deoA* in *E. coli*) together with *deoC* (deoxyribose-5-P → glyceraldehyde-3-P + acetaldehyde), while the other consists of *pup* (*deoD* in *E. coli*) together with *deoB* ((d)Ribose-1-P → (d)Ribose-5-P). If a physiological rationale behind the physical clustering should be extracted, then breakdown of purine ribonucleosides (AR, IR, and GR) utilizes enzymes solely from the *pup-deoB* unit, while breakdown of all other nucleosides requires enzymes from both units (Fig. 13 and Tables 1 and 3). It should further be noted that several of the salvage genes found at the preserved positions in *S. thermophilus* seem to be disrupted (e.g., *xpt*, *pdp*, and *deoC*). Lack of a functional *deoC* gene has previously been found by enzyme analysis in 3 out of 4 strains of *S. thermophilus* [142]. Very scarce data are available with regard to the regulation of gene expression of the salvage enzymes in *L. lactis*. In contrast to the findings in *E. coli* and *B. subtilis*, addition of nucleosides to the growth medium of lactococci does not seem to induce the salvage enzymes Pdp, Pup or Cdd and nucleosides have been found not to function as sole energy sources (K. Hammer and J. Martinussen, unpublished). DNA microarray profiling of *L. lactis* MG1363 indicates that heat shock induces expression of the *upp*, *dukA*, and *udp* genes 5-, 22-, and 23-fold, respectively (M. Kilstrup and A. Nielsen, unpublished). This should increase uracil incorporation into UMP (*upp*) and conversion of NdR to dNMP (*dukA*). The *udp* gene may encode either a uridine phosphorylase or a purine nucleoside phosphorylase. In both cases the enzyme activity would result in degradation of an NR to the free nucleobase (Figs. 2, 3, and 13).

10.9. Nucleoside salvage systems with trans-N-*deoxyribosylases*

With respect to their nucleotide metabolism lactobacilli may be divided into two groups, dependent on whether or not they require deoxyribonucleosides for growth [137]. The requirement is most likely caused by nonfunctional ribonucleotide reductases, but many strains are also auxotrophic for both purines and pyrimidines due to missing biosynthetic pathways for UMP and IMP [143–145]. The salvage pathways from the deoxyribonucleoside auxotrophic lactobacilli were found to differ from most other bacteria by the presence of *trans-N*-deoxyribosylases. These industrially important enzymes, which catalyses the exchange of nucleobase between a deoxyribonucleoside and a free nucleobase, were first discovered in lactobacilli. As shown in reaction 8 in Fig. 14, the free nucleobase N^1 is exchanged with the N^2 nucleobase in the N^2dR substrate, resulting in the formation of a new product N^1dR. An accompanying feature of the NdR requiring lactobacilli is a total lack of nucleoside phosphorylases, which would otherwise degrade the required NdR by cleavage to the free base and dRibose-1-P (Fig. 13). The growth requirements of the NdR requiring lactobacilli are satisfied if they are supplied with any deoxyribonucleoside, and purine and pyrimidine bases.

Two classes of *trans-N*-deoxyribosylases with different substrate specificities have been characterized: Purine–transdeoxyribosylases (PTD) only use substrates containing a purine base, while the *N*-deoxyribosylases (NTD) use substrates containing both purine and pyrimidine bases [146]. The three-dimensional structure of these enzymes has been solved [147]. The presence of a PTD enzyme and the simultaneous lack of purine nucleoside phosphorylase was demonstrated by enzyme assays from a series of lactic acid bacteria [148]. Almost all lactobacilli had this type of salvage system (12 out of 15 tested), as well as *Leuconostoc mesenteroides*, *Pediococcus* and *Aerococcus viridans*. *Lb. casei* subsp. *rhamnosus* had purine nucleoside phosphorylase activity and no PTD enzyme, like the salvage system found in lactococci, while *Lb. plantarum* and *Lb. casei* subsp. *casei* had both enzyme activities, indicating that they may contain an intermediate type of salvage pathway system. The presence of a nucleoside phosphorylase suggests that these lactobacilli do not have requirements for deoxyribonucleosides. In fact both species are prototrophic for both ribo- and deoxyribonucleosides [145].

10.10. Deoxyribonucleoside kinases in deoxyribonucleoside requiring lactobacilli

The utilization of all four deoxynucleosides is achieved by the action of deoxyribonucleoside kinases (reaction 3, Fig. 13). While most bacteria have a thymidine kinase, only few genera such as *Lactobacillus* and *Bacillus* have experimentally been shown to possess all four deoxyribonucleoside kinase activities [141]. From the analysis of genome sequences many of the lactic acid bacteria, including *L. lactis* and the pathogenic streptococci appear to share this property. *Lb. acidophilus* harbors three different NdR kinases: Tdk, an AdR/CdR kinase and an AdR/GdR kinase. The two latter proteins are heterodimers sharing a common subunit with AdR kinase activity, encoded by the *dak* gene. Remarkably, the second subunit of the two separate enzymes also originates from a single gene, *dgk*. From the published data it appears that the small amino acid difference in the N-terminal end (TV versus M) is due to different translational start sites [141]. The *dgk* gene is located downstream of the *dak* gene and they most likely form an operon in *Lb. acidophilus* [141].

10.11. Ribonucleoside salvage in deoxyribonucleoside requiring lactobacilli

The general outline of the salvage pathways in lactobacilli are given in Fig. 14. Salvage of nucleobases cannot occur by means of nucleoside phosphorylases, since they are not found in this group of lactobacilli. Instead, ribonucleoside hydrolases have been identified by protein purification (reaction 7). The hydrolase enzyme from *Lb. delbrueckii* has activity towards both purines and pyrimidine substrates including cytidine, but importantly, no activity towards the corresponding deoxyribonucleosides was found [149]. The free nucleobase formed by the nucleoside hydrolase (Fig. 14, reaction 7) may subsequently be converted either to the corresponding deoxyribonucleoside by *trans-N*-deoxyribosylase (reaction 8) or by the corresponding phosphoribosyl transferase to the corresponding NMP (reaction 1). PRPP, the other substrate for this reaction, is provided by the enzyme PRPP synthase. The Ribose formed by the nucleoside hydrolase, may be used as a source of energy or carbon, and Ribose-5-P is also a substrate for PRPP synthase. The pyrimidine ribonucleosides may also be salvaged by conversion to the corresponding NMP through the action of a cytidine/uridine kinase, as in lactococci (reaction 3 in Fig. 14).

11. Mutant phenotypes

Many lactobacilli are reported to have requirements for either purines or pyrimidines or both. The genome sequence of *Lb. johnsonii* (formerly *Lb. acidophilus*) shows a complete lack of genes encoding the purine biosynthetic enzymes needed for IMP synthesis [143] (Fig. 2). However, the presence of relevant genes in the genome sequences of lactic acid bacteria is not always an

indication of prototrophy, since genes may have been inactivated by mutations, giving rise to requirements for purines or pyrimidines. Pyrimidine and deoxyribonucleotide requirements are also found in strains of *Lb. johnsonii* as evidenced by growth studies [55,150]. Purine and pyrimidine requirements have also been well documented in several strains of *Lb. sake* [144]. In many lactobacilli the pyrimidine requirement may be satisfied by the pyrimidine precursor, orotate, which is present in milk [151]. However, orotate utilization depends on the presence of functional PyrE and PyrF enzymes (Fig. 3), as well as a transport system for orotate. Furthermore, the pyrimidine defect in many lactobacilli seems to be located in the first step in the biosynthesis pathway, giving requirement for both uracil (orotate) and arginine [152].

In the prototrophic lactococci, a large number of mutants have been isolated in the purine and pyrimidine pathways and the auxotrophic phenotypes have been determined (Table 4). Spontaneous and transposon-induced mutations have been obtained by selection for mutant phenotypes [9,132], and site-directed mutants have been obtained by in vitro construction of the mutant DNA followed by homologous recombination into the chromosome [115]. Mutations in the genes of the de novo pathways usually result in auxotrophy, which can be satisfied with either nucleobases or nucleosides.

The relevant phenotypes of the available pyrimidine de novo mutants are shown in Table 4. Because the rich GM17 broth contains sources of pyrimidine nucleobases and nucleosides, *pyr* and *car* mutants show no Pyr phenotypes in this medium. However, no sources of pyrimidines or purines are present in SA medium, which is why *carA*, *carB*, *pyrB*, *pyrF* and most probably also *pyrC* and *pyrE* mutants cannot grow in this medium without addition of at least one source of pyrimidines or a preformed intermediate. The source can be U, UR, CR, UdR or CdR as seen in Table 5, but *carA* and *carB* mutants additionally have the possibility to grow in the absence of pyrimidines if arginine is added as a source of carbamoyl phosphate (CP) [14], the product of the CarAB enzyme. Supply of CP from arginine requires the action of the ArcA and ArcB enzymes from the arginine deiminase pathways [153]. Because *L. lactis* harbors two functional *pyrD* genes, the disruption of neither *pyrDa* nor *pyrDb* leads to a Pyr^- phenotype [21]. Likewise, a *pyrK* mutant that lacks the NAD^+ binding subunit of the PyrDbK holoenzyme has no Pyr^- phenotype [21]. Interestingly, a *pyrDa*, *pyrK* double mutant has a leaky pyrimidine requirement, making it interesting for regulatory studies. Mutants in *pyrG* have phenotypes different from the de novo mutants because the synthesis of only CTP is affected. This deficiency can only be satisfied by addition of CR and requires an intact *udk* gene. Removal of CR by the cytidine deaminase enzyme (encoded by the *cdd* gene) is insignificant so a *pyrG cdd* double mutant grows as well on CR as the *pyrG* mutant, albeit with a lower growth yield. GM17 broth apparently contains inhibitory substances for utilization of CR, since 100-fold higher concentration is needed in GM17 than in GSA, as seen in Table 4. This might be caused by purine nucleosides or UR, which have been shown to inhibit transport and conversion to CMP respectively [8]. Thymine uptake in GM17 might also be inhibited in a *thyA* mutant since the required enzyme Pdp also has uridine as substrate. This

Table 4
Phenotypes of *L. lactis* subsp. *cremoris* MG1363 mutants affected in the purine and pyrimidine de novo pathways

Mutant	General description	Phenotype in GM17 medium[a]	Phenotype[c] in GSA medium[b] or milk[d]	Reference
carA	Pyrimidine de novo	+	Pyr^-[d]	[15]
carB	Pyrimidine de novo	+	Pyr^-[d]	[14]
pyrB	Pyrimidine de novo	+	Pyr^-[d]	[15]
pyrDa	Pyrimidine de novo	+	+	[20]
pyrDb	Pyrimidine de novo	+	+	[21]
pyrK	Pyrimidine de novo	+	+	[21]
pyrK pyrDa	Pyrimidine de novo	+	Leaky Pyr^-[d]	[21]
pyrF	Pyrimidine de novo	+	Pyr^-	[21]
pyrG	CTP synthesis	Requires CR_{1000}	Requires CR_{20}	[43]
thyA	dTMP synthesis	Requires thymidine (TdR_5)	Requires thymidine (TdR_{20})	[165]
purD	Purine de novo	+	Pur^-	[9]
purR	Purine de novo	+	Pur^-	[9]
guaA	GMP Branch	Partial requirement for G, GR, or GdR	Requirement for G, GR, or GdR	[2,138]

[a] M17 medium with glucose [166]. + symbolizes no observable phenotype.
[b] SA medium with glucose [167].
[c] Pyr^- phenotype: requires a source of pyrimidines, i.e., uracil (U_{10}), uridine (UR_{20}), cytidine (CR_{20}), deoxyuridine (UdR_{20}), and deoxycytidine (CdR_{20}). Pur^- phenotype: requires a source of purines, i.e., guanine (G_{15}), adenine (A_{15}), hypoxanthine (Hx_{15}), xanthine (X_{15}), guanosine (GR_{30}), adenosine (AR_{30}), inosine (IR_{30}), deoxyguanosine (GdR_{30}), deoxyadenosine (AdR_{30}), or deoxyinosine (IdR_{30}). The subscript indicates $\mu g\,ml^{-1}$ usually added of the compound.
[d] Because milk does not contain sources of purines or pyrimidines except for orotate, the mutant phenotypes are usually similar in milk and GSA medium. However the *pyr* mutants labeled[d] would have Pyr^+ phenotype in milk if they possessed an orotate transport system.

could also explain the lack of growth in GM17 as previously observed [140].

Because mutations in pyrimidine salvage genes may severely affect the source of pyrimidine to be used to satisfy a pyrimidine requirement, we have included a description of which pyrimidine sources are expected to support growth of various double mutants in Table 5. Only the *pyr upp* mutant has been constructed, but the growth phenotypes from the other double mutants may be deduced from the established salvage pathways in lactococci. The various salvage routes leading to nucleotide synthesis have been discussed in the salvage section, so the growth phenotypes can be inferred from Fig. 3.

A mutation in the *thyA* gene (Table 4) renders the cell unable to form thymine nucleotides de novo. However, due to the presence of the Tdk enzyme, a *thyA* mutant can obtain dTMP from thymidine (TdR). Because this reaction is required for dTMP synthesis in a *thyA* mutant, a *thyA tdk* double mutant is non-viable. Interestingly, the deprivation of thymidine from a *thyA* mutant not only leads to growth stasis, but actually leads to rapid cell death [140] in a process originally termed thymine-less death [154]. Because the intracellular concentration of deoxyribose-1-P is sufficiently high to allow the Pdp enzyme to convert U to UdR, the same enzyme should allow for TdR formation from T. A *pdp* mutation in strain CHCC373 seems to be the cause of the lack of growth of the *thyA* mutant in this strain on thymine (K. Hammer unpublished).

Only few purine de novo mutants have been analyzed. In accordance with the pathways in Fig. 2, all purine auxotrophic mutants could grow at wild-type rates with a large number of purine sources: A, G, Hx, X, AR, IR, and GR. The list of good purine sources has been extended, using a *purD*::IS*S1* mutant [9], to include the deoxyribonucleosides AdR, GdR, and IdR (Table 4). Xanthosine (XR) can also support the growth of the *purD* mutant, albeit at a lower rate, suggesting that either the uptake or the conversion of XR to XMP is inefficient. Most purine auxotrophic mutants have been obtained by ampicillin counterselection after chemical or transposon-induced mutagenesis [132] [9]. From IS*S1* mutagenesis, two *purD* and one *purL* mutant were identified [9].

Mutations in the *guaA* gene have been obtained as both *arl* (acid resistant locus [2]) and *trm* mutants (temperature resistant mutant [3]), after pGh9:IS*S1* mutagenesis of *L. lactis* MG1363 or its *recA* derivative VEL1122. The mutants grew slowly on rich GM17 medium, and the partial growth defect was eliminated by addition of either G or GR, but not by any other purine source. A similar pGh9:IS*S1* mutant of *L. lactis* MG1363 could only grow in the presence of G, GR or GdR in the chemically defined SA medium (K. Hammer and M. Kilstrup, unpublished). This shows that the apparent leaky phenotype of the *guaA* mutant in unsupplemented GM17 medium is due to a limited supply of G, GR, or GdR in this medium, or possibly to low uptake rate due to competition for transport and catabolism from the other bases and nucleosides (see below in the section on nucleotide pools).

The stress phenotypes of the *guaA* mutants are extremely interesting. The mutants were obtained by selection for increased growth at 37.5 °C on solid GM17 medium adjusted to pH5 (*arl* mutants [2]), or at 39.3 °C on neutral GM17 plates (*trm* mutants [3]). The insertions spanned the entire *guaA* gene, so the phenotypes are all consistent with an inactivation of the GuaA enzyme and a resulting GTP (or either GMP or GDP) limitation in GM17 medium. The mutants had 10-fold increased survival compared to the wild-type strain during oxidative stress (1 mM H_2O_2 in 30 min), and likewise during heat shock (55 °C for 5 min). The increased survival, relative to the wild-type became even more pronounced when the cells were pre-adapted (at 37 °C for 15 min) prior to heat shock. All stress phenotypes disappeared when G or GR was added, suggesting that guanine nucleotide limitation is the stress signal [2,3]. Decoynine, which inhibits the GuaA enzyme, resulted in the same phenotype. The *guaA* mutant also showed increased resistance to glucose starvation under acid conditions and long term survival during glucose starvation, at either acidic or neutral conditions. A *relA* deletion, which resulted in the formation of a truncated protein that synthesized constitutive high levels of (p)ppGpp under unstressed conditions, showed the same stress phenotype [2]. This phenotype, however, did not disappear in the presence of G or GR. The highly elevated flux from GTP to (p)ppGpp in the *relA* strain appeared to result in lowered intracellular pools of GTP, but the actual stress signal has not yet been identified [2]. GTP limitation is a general alarm signal in most organisms. In *B. subtilis* GTP starvation induces sporulation [155], while it results in differentiation of aerial mycelium formation in *Streptomyces coelicolor* [156].

Mutants in the pyrimidine salvage genes are easily selected and recognized by their resistance to 5-fluoro-derivatives of either uracil (FU), uridine (FUR), cytidine (FCR), deoxyuridine (FUdR), or deoxycytidine (FCdR). The 5-fluoro-derivatives become toxic if they are converted to the dUMP derivative FdUMP because this compound mimics dTMP and strongly inhibits the thymidylate synthase [157]. The 5-fluoro-derivatives are, with few exceptions, considered indistinguishable from the un-fluorinated compounds to the cell, so conversion routes identified with the fluoro-derivatives are directly extrapolated to include the native compounds.

Mutants in *upp* are resistant to FU concentrations below 0.3 μg ml^{-1} ($FU_{0.3}$) (Table 5), because the conversion to FUMP is prevented (Fig. 3). Higher FU concentrations, in combination with the high internal

Table 5
Growth characteristics of salvage mutants in *L. lactis* subsp. *cremoris* MG1363 on SA medium[a] supplemented with glucose (data from [133] and Hammer, Martinussen and Kilstrup, unpublished)

Salvage geno-type	A pyrimidine source: *pyr* auxotrophic mutants[b]					A toxic fluor analogue with or without uracil (U) and/or purine nucleosides (AR or GdR): pyrimidine prototrophs[c]						
	U	UR	CR	UdR	CdR	$FU_{0,3}$	FU_5	$FUdR_{10} + U_{30}$	$FUR_{10} + U_{30}$	$FCdR_{0,5}$	$FU_2 + U_6 + GdR_{500}$	$FU_{10} + AR_{500}$
Wild-type	+	+	+	+	+	–	–	–	–	–	–	–
upp	–	+	+	–	–	+	–	–	–	–	–	–
upp pdp	– *	+*	+*	– *	– *	+	+	–	–	–	+*	+
pdp[d]	+ *	+ *	+*	– *	– *	– *	– *	– *	– *	– *	+	– *
pup[d]	+*	+*	+*	+*	+*	– *	– *	– *	– *	– *	+	–*
upp, tdk	– *	+*	+*	– *	– *	+	+	+	–	+	+	–
tdk	+*	+*	+*	+*	+*	–	–	+	–	–	+	–
upp pdp tdk	– *	+*	+*	– *	– *	+	+	+	–	+	+	+
upp pup tdk	– *	+ *	+ *	– *	– *	+	+	+	–	+	+	+
udk	+*	+*	+*	+*	+*	–	–	–	+	–	–	–
upp udk	Non viable					+	–	–	+	–	–	–
cdd	+*	+*	?	+*	– *	–	–	–	–	+	–	–
upp cdd	– *	+*	?	– *	– *	+	–	–	–	+	–	–

+ indicates growth and – no growth. * indicates expected growth phenotype (the experiment has not been performed). Number indicates μg ml^{-1} used in the plates.

[a] SA medium [167].

[b] Cytosine is not tested, since it cannot be used as a pyrimidine source in Lactococci.

[c] Subscripts indicate μg ml^{-1} used in the plates.

[d] Studied in a purine auxotroph mutant (*purD*).

deoxyribose-1-P concentration (see chapter about salvage), drive the Pdp reaction towards FUdR synthesis, so a *upp* mutant is sensitive to FU_5. Both *upp pdp* and *upp tdk* mutants are resistant to FU_5 (Table 5). The *upp pdp* mutant is resistant because it is unable to convert FU to FUdR and the *upp tdk* mutant because it cannot convert FUdR to the toxic FdUMP. The Pdp reaction can also be reversed by increasing the intracellular level of Ribose-1-P. This is done by addition of AR, which is cleaved by the Pup enzyme to A and Ribose-1-P. As expected the *upp* mutant is sensitive to $FU_{10} + AR_{500}$, and the *upp pdp* double mutant is resistant. This condition thus provides a selection criterion for obtaining *pdp* mutants in a *upp* background.

A *pdp* single mutant has a subtle phenotype; i.e., it is resistant to $FU_2 + U_6 + GdR_{500}$ (Table 5), which has been used for its selection among wild-type cells. If U_6 is added together with FU_2 as a competitive substrate for the Upp enzyme, the amount of FUMP is reduced considerably. It appears that Upp has a preference for the native substrate over the 5-F-derivative, which is not the case for other enzymes of the pathways. Thus, addition of FU + U specifically counteracts the Upp-dependent toxicity, simulating the phenotype of a *upp* mutant. GdR is added to increase the internal pool of dRibose-1-P to reverse the Pdp reaction towards FUdR production. Just as *pdp* mutants are resistant to $FU_2 + U_6 + GdR_{500}$, so are *pup* mutants because the increased dRibose-1-P production from GdR is dependent on Pup.

A *tdk* single mutant is resistant towards $FUdR_{10} + U_{30}$ because (i) the mutation prevents the conversion to FdUMP, and (ii) the Upp-dependent toxicity of the FU produced by the Pdp dependent phosphorolysis is counteracted by competition with U. The *tdk* mutant is resistant towards $FU_2 + U_6 + GdR_{500}$ for the same reasons. By itself a *tdk* mutants is sensitive to FCdR (Table 5) because it is deaminated by the Cdd to FUdR, followed by phosphorolysis to FU by Pdp, and subsequently converted to FUMP by Upp. The *upp tdk* double mutant described above is impaired in the last reaction and is resistant to $FCdR_{0.5}$. A *upp pdp tdk* and a *upp pup tdk* triple mutant exhibits the combined resistances of the individual mutants (Table 5).

A *udk* mutant is resistant to $FUR_{10} + U_{30}$ because (i) it is unable to convert FUR to FUMP, and (ii) U is added as a competitor against Upp-dependent FU toxicity. A *upp udk* double mutant has the combined resistances of the *upp* and *udp* mutants.

A *cdd* mutant is resistant to $FCdR_{0.5}$ (i) because it is unable to convert it to FUdR, and (ii) because the conversion to FdCMP requires high FCdR concentrations (e.g., $FCdR_2$), indicating the presence of a CdR kinase. A *upp cdd* double mutant have the combined resistances of the *upp* and *cdd* mutants.

12. Manipulation of NTP pools in *L. lactis*

Nucleotides play a key role in the control of many aspects of cell physiology, and changes in nucleotide pools will affect cell performance in a number of ways. Also, when studying gene expression both at the single gene

level and at the global level using proteomics and transcriptomics, manipulation of nucleotide pool sizes is an important way to simulate the challenges the cells meet in the environment. Nucleotide pools can be manipulated, by using strains with a requirement for purines or pyrimidines and growing them in the presence of different combinations of nucleotide precursors. Purine nucleotide pools may be increased or decreased compared to the wild-type levels. Addition of purine bases to the reference strain *L. lactis* MG1363 leads to an increase in GTP and ATP concentrations accompanied by a small increase in growth rate (Table 6). As mentioned in the introduction, this suggests that *L. lactis* MG1363 has a partial purine requirement [8]. A reduction in purine pool sizes can instead be obtained by growing a purine-requiring strain in the presence of inosine and excess cytidine (Table 6). Since inosine and cytidine, as previously described, share a common uptake system, cytidine acts as a competitive inhibitor of inosine uptake. The advantage of this method over purine depletion is that the culture can grow at steady state. Purine depletion can be obtained by growth of a purine-requiring strain in a limited amount of exogenous purines [4]. As mentioned above, GTP limitation can be obtained by inhibition of GuaA by decoynine [4].

Pyrimidine limitation may also be obtained in several ways, using either a *pyr* auxotroph or a bradytroph. Growth of a *carB* mutant in a pyrimidine-free medium with an excess of arginine results in reduced growth rates and a reduction of CTP and UTP pool sizes (Table 6). Carbamoyl phosphate is normally produced by the CarAB enzyme, which is inactivated in a *carB* mutant. However, *L. lactis* can degrade arginine via the arginine deiminase pathway, which forms carbamoylphosphate as an intermediate [153]. Arginine, thus, serves as a donor of carbamoylphosphate, which is subsequently utilized in pyrimidine biosynthesis [14]. Pyrimidine limitation can also be obtained in a *pyrB* strain by growing it with cytidine as pyrimidine source in the presence of inosine. As previously described, inosine inhibits cytidine uptake resulting in a reduced growth rate. Purine pool sizes are found to be slightly elevated, whereas the UTP pool is low, as expected. Surprisingly, the CTP pool was increased five times over the wild-type level, which appears controversial under pyrimidine limiting conditions. If however, the Cmk enzyme has a higher affinity for cytidine than the Cdd enzyme, then conversion to CMP would be favored over the deamination to uracil under low cytidine concentrations, leading to the imbalance in CTP and UTP pools [8].

The CTP pool size may be manipulated in several ways. Restricting cytidine uptake in a *pyrG* strain by the simultaneous addition of cytidine and inosine leads to a decrease in the CTP pool size by one order of magnitude [8]. Under these conditions the purine pools are increased because the reduced rate of RNA synthesis due to a limited CTP supply diminishes the drain of purine nucleotides. The simultaneous addition of uridine and cytidine to a *pyrG* strain also reduces the CTP pool by one order of magnitude [48]. This is explained by the fact that uridine acts as a competitive inhibitor of uridine kinase, the enzyme responsible for the phosphorylation of both uridine and cytidine to the corresponding NMP (Fig. 3). A third way of manipulating the CTP pool size was by constructing a series of strains with different constitutive levels of *pyrG* expression through insertion of synthetic promoters with different strengths upstream of *pyrG*. The CTP pool size was dependent on *pyrG* expression and varied between 10% and 250% compared to the wild-type level [50].

13. Genetic tools derived from nucleotide metabolism

13.1. Food grade marker genes

Auxotrophic *pyr* and *pur* mutants require bases or nucleosides in both defined medium and in milk (Table 4). This property makes *pur* and *pyr* genes powerful candidates for food grade marker genes in

Table 6
Effects of the presence of different nucleotide precursors on nucleotide pool sizes in purine, pyrimidine, and cytidine requiring strains of *L. lactis* subsp. *cremoris* MG1363[a]

Genotype	T_D[b] (min)	Addition[c]	Pool sizes – nmol per mg dry weight			
			GTP	ATP	CTP	UTP
Wild-type	56	–	0.8	4.0	0.8	1.6
Wild-type	49	$A_{15} + Hx_{15} + GR_{30}$	2.9	7.9	0.5	1.7
pyrG, *cdd*	112	$CR_{20} + IR_{100}$	4.1	8.7	0.1	4.8
purD::IS*S1*	91	$IR_{20} + CR_{2000}$	0.9	3.1	1.3	1.7
pyrB::IS*S1*	174	$CR_{20} + IR_{100}$	1.9	5.0	4.2	0.6
carB::IS*S1*	188	–	1.0	5.1	0.5	0.9

[a] Table obtained from [8] with permission from the American Society for Microbiology.
[b] T_D is generation time in minutes.
[c] All experiments were conducted at 30 °C in SA medium with glucose, nucleosides, and nucleobases added. The final concentrations are shown as subscripts in μg ml^{-1}. A, adenine; Hx, hypoxanthine; GR, guanosine; CR, cytidine; IR, inosine.

genetic engineering of dairy strains. The major drawback of using biosynthetic genes as selectable markers is that a corresponding mutation is required in the recipient strain. Chromosomal amber mutations in *pyrF* and *pur* genes have also been used in recipient strains for selection of plasmids that carry a food grade nonsense suppressor, which reverts the auxotrophy of the mutants [6].

Because *upp* is required for the conversion of both uracil and FU, it can be used as a selectable marker in selections for Upp^+ transformants as well as in selections for loss of the same plasmid, by the Upp^- phenotype of the plasmid cured strains. Both selections require a *upp* mutation in the host strain. When used in the forward selection for the presence of the *upp* gene, selections are made for growth on uracil as pyrimidine source. Here the host needs to be a pyrimidine auxotroph. On the other hand, the FU resistance of a *upp* mutant in a pyrimidine prototroph enables selection for loss of a functional upp^+ gene. This method has been used as an efficient genetic tool for monitoring deletions [158].

A lactococcal plasmid, pDBORO, isolated from the *L. lactis* subsp. *lactis* biovar. *diacetylactis* strain DB0410, turned out to encode a transport system responsible for the uptake of orotate, the fourth intermediate in the pyrimidine biosynthetic pathway (Fig. 3). The gene was named *oroP* and was shown to enable growth of a pyrimidine auxotrophic mutant with orotate as the sole pyrimidine source. Morover, *oroP* conferred sensitivity towards the pyrimidine-analog 5′-fluoroorotate (E. Defoor and J. Martinussen, unpublished). The *oroP* gene has been demonstrated to be an excellent tool that can be used both as a selection marker in a pyrimidine requiring strain and as a counter-selection marker in the presence of 5-fluoroorotate. In the latter case a number of strains can be used directly, including *L. lactis*. The system thus has the benefits of the *upp* selection system without the need for a mutation in the recipient. The *oroP* system has been shown to work in a number of microorganisms, including *E. coli* and *B. subtilis*, either directly in the wild-type strains or in strains impaired in orotate uptake [159]. The usefulness of the system has been demonstrated in the construction of a number of strains containing synthetic promoters upstream of *tpi*, encoding triosephosphate isomerase (C. Solem, E. Defoor, J. Martinussen and P. Ruhdal-Jensen, unpublished).

The potential of *thyA* as a food grade marker was proposed in 1990 by Ross et al. [160], who cloned and sequenced the gene but its use as a marker gene in lactic acid bacteria was not investigated. In lactococci and several other lactic acid bacteria, *thyA* mutants cannot be directly selected for, because many species are naturally resistant to drugs such as trimethoprim, which are usually used for their selection [68,161]. However, *thyA* mutants have been isolated by reverse genetics, and these were found to require thymidine even in rich medium [115].

13.2. Containment systems and phage resistance mechanisms

Mutants lacking a functional *thyA* gene were found to die "the thymineless death" when deprived of thymidine [115]. This property has been used in a system for containment of genetically engineered strains with recombinant genes, by inserting the human interleukin 10 gene into the *thyA* locus [140].

Lactococci and probably other lactic acid bacteria become phage resistant when starved for nucleotides, including thymine. Thymine starvation is particularly interesting since it only prevents DNA synthesis but allows RNA synthesis, leaving the cells capable of performing most of the metabolic processes, including production of lactic acid [115,162].

13.3. Conditionally regulated gene expression systems

PurR-regulated promoters are regulated around 30-fold by purines [86]. In media containing purine bases or nucleosides, the promoters allow very little transcriptional initiation. If the cells are transferred to a medium without purine bases such as milk, these promoters become active. The promoters can therefore be used for specific expression of recombinant proteins in milk [163].

14. Concluding remarks

The knowledge of the physiology and genetics of nucleotide metabolism is important in many ways. The results from genome projects and the post genomic research should now in principle allow for quantitative systems biology for establishing a computer model describing how nucleotide metabolism is controlled within the metabolic framework of the living cell. In order for such a model to be accurate it is important to take into account the various regulatory mechanisms working on the metabolic as well as on the level of gene expression as exemplified by studies of *pyrG* regulation and the size of the CTP pool [50].

With respect to regulation of expression of nucleotide biosynthetic genes, two major regulons are found in almost all investigated lactic acid bacteria: the purine and pyrimidine regulons, which are controlled by the regulatory proteins PurR and PyrR, respectively. PurR is responsible for regulation of the gene products involved in the IMP biosynthetic pathway, as well as some involved in purine salvage and in folate metabolism. PyrR is responsible for the regulation of the gene products involved in the UMP biosynthetic pathway. It should be

noted that by using global methods such as proteomics and a partial genomic DNA array only few gene products not related to the mentioned pathways have been identified so far. The conditions used in *L. lactis* have been a high UMP as well as PyrR mutants giving low and high Pyr enzyme levels, respectively. However, mutants in *purR*, resulting in constitutive levels of the proteins belonging to the regulon are also needed for global investigations in lactic acid bacteria such as it have been performed in *S. pneumoniae* [87]. Physiological conditions giving altered nucleotide pool sizes are also expected to give favorable information in future physiological studies as well as global studies on gene expression.

With respect to gene regulation, PurR is acting on initiation of transcription either as a repressor or as an activator dependent on the organism, and PyrR is a specific RNA binding protein involved in attenuation of transcription, while RNA polymerase alone is involved in the attenuation of pyrG. Other regulatory mechanisms are expected to be identified in lactic acid bacteria either affecting gene products in the nucleotide metabolism or having nucleotide derivatives as effectors. It also remains to be seen if examples of the riboswitch mechanism should be found in lactic acid bacteria, as suggested for the control of the *xpt* gene in *L. lactis* [164].

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